GENETIC ABLATION OF *Ccr6* CONFERS DIFFERENTIAL EXACERBATION IN A SPONTANEOUS COLITIS MODEL

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Dedication

To my beautiful family

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Genetic Ablation of *CCR6* Aggravates Colitis in a Spontaneous Colitis Model

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***CCR6* Deficiency Aggravates Colitis in a Spontaneous Mouse Model of Colitis**

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ABBREVIATIONS

αvβ8	Alpha v beta 8 integrin
APCs	Antigen-presenting cells
bp	Basepairs
CCL20	C-C-Chemokine ligand 20
CCR6	C-C-Chemokine receptor 6
CD	Crohn's disease
DCs	Dendritic cells
DSS	Dextran sodium sulphate
Eef2	Eukaryotic translation elongation factor 2
ENU	N-ethyl-N-nitrosourea
ER stress	Endoplasmic reticulum stress
EAE	Experimental autoimmune encephalitis
FAE	Follicle-associated epithelium
FOXP3	Forkhead box transcription factor 3
gDNA	Genomic DNA
GWAS	Genome-wide association studies
H&E	Haematoxylin and eosin
IBD	Inflammatory bowel diseases
IEL	Intraepithelial lymphocyte
ILFs	Isolated lymphoid follicles
IFN-γ	Interferon gamma
IL	Interleukin
KO	Knockout
LARC	Liver activation-regulated chemokine

LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
Muc2	Mucin 2 gene
M cell	Microfold cell
MALT	Mucosal-associated lymphoid tissue
MLNs	Mesenteric lymph nodes
MIP-3α	Macrophage inflammatory protein – 3 alpha
NF-κB	Nuclear factor-κB
NH_4Cl	Ammonium chloride
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PRRs	Pattern recognition receptors
PBS	Phosphate buffered saline
PPs	Peyer's patches
qPCR	Quantitative polymerase chain reaction
RORα	RAR-retinoic acid-related orphan receptor alpha
RORγt	RAR-retinoic acid-related orphan receptor gamma
SAMP1	Senescence-accelerated mouse P series
SCID	Severe combined immune-deficiency
SED	Subepithelial dome
SNP	Single nucleotide polymorphism
SPF	Specific pathogen-free
STAT3	Signal transducer and activator of transcription 3
TCR	T-cell receptor
TGF-β1	Transforming growth factor beta1
TNBS	2, 4, 6-trinitrobenzene sulfonic acid

TNF-α	Tumour necrosis factor alpha
UC	Ulcerative colitis
UPR	Unfolded-protein response
WT	Wild type

ABSTRACT

Inflammatory bowel disease (IBD) is an immunological imbalance of the gut, characterised by chronic inflammation involving the mucosal lining of the colon. IBD presents in two forms: Crohn's disease (CD) and ulcerative colitis (UC). The increasing global incidence of IBD has necessitated a better understanding of the underlying mechanism of disease progression, and while its aetiology remains obscure, current literature suggests that both genetic susceptibility and environmental aspects have an influence on the course of IBD.

Murine models have led to a number of discoveries in IBD pathogenesis. Amongst the many mouse models developed, the Winnie mouse model of colitis closely resembles the condition of human colitis in terms of both symptoms and immunology. A number of studies have reported that Winnie mice are a preclinical resource for better understanding IBD pathogenesis. An emerging mechanism for the pathogenicity of IBD development includes a dysregulated chemokine system. In particular, the CCR6-CCL20 axis has been implicated due to its major role in regulating T cell homeostasis in the intestine. The current literature regarding CCR6-CCL20 is inconclusive as to the exact role of CCR6.

The aim of this study was to introduce CCR6 deficiency in the Winnie spontaneous colitis model in order to further elucidate the functional role of CCR6 in colitis and characterise clinical, histopathological and immunological changes.

In this present study, the hypothesis was that the genetic ablation of the *Ccr6* gene would exacerbate intestinal inflammation.

Winnie mice were backcrossed with *Ccr6*$^{-/-}$ mice to generate Winnie mice with a *Ccr6* deficiency. Winnie x *Ccr6*$^{-/-}$ mice developed normally and did not have any developmental abnormalities. At around 8–12 weeks of age, the Winnie x *Ccr6*$^{-/-}$ mice were clinically assessed, and no significant differences were noted between Winnie and Winnie x *Ccr6*$^{-/-}$ mice with regard to body weight. There was, however, a significant increase in the colon weight/body weight ratio (±0.026 mg/g), with Winnie x *Ccr6*$^{-/-}$ mice revealing a significant increase in colon weight by body weight ratio compared to control mice (WT and *Ccr6*$^{-/-}$ mice) of a similar magnitude to the expected Winnie-only phenotype.

Histopathological examination of proximal and distal colonic sections showed no obvious inflammatory changes in the control mice (WT grade-0 and *Ccr6*$^{-/-}$ grade-0). Winnie mice demonstrated the expected amount of inflammation in the distal colonic segment (grade-6). Surprisingly, there was a differential distribution of inflammation in Winnie x *Ccr6*$^{-/-}$ mice, where the proximal colon revealed significant inflammation (grade-8) while there was mild inflammation (grade-4) in the distal colon.

Immuno-phenotyping of splenocytes and colonic lymphocytes did not reveal any significant changes in the functions of T regulatory cells. To identify the differential regulation in Winnie x *Ccr6*$^{-/-}$ mice, we cultured ex vivo colonic segments from Winnie, Winnie x *Ccr6*$^{-/-}$, *Ccr6*$^{-/-}$ and WT mice to assess the secretion of cytokines. Interestingly, molecular and cytokine assays demonstrated elevated levels of anti-inflammatory cytokine interleukin-10 (IL-10) in the distal colon and increased expression of pro-inflammatory cytokine, interleukin-1β (IL-1β), in the proximal colon.

In Winnie x *Ccr6*$^{-/-}$ mice, an increased expression of IL-1β in the proximal colon correlated with an exacerbation of inflammation. Increased expression of anti-

inflammatory cytokine IL-10 was associated with an amelioration of inflammation in the Winnie x *Ccr6*$^{-/-}$ distal colon. These results suggest that absence of the *Ccr6* gene selectively confers amelioration of colitis in the distal colon but exacerbation in the proximal colon.

Overall, this study presents significant and novel findings in the CCR6-deficient spontaneous colitis model. Genetic ablation of *Ccr6* conferred regional differential exacerbation between colonic segments of Winnie x *Ccr6*$^{-/-}$ mice. Findings from this study reveal the hitherto unknown mechanism of *Ccr6* differential distribution in the colon, thereby demonstrating an important role for CCR6 in the development of colitis. This will throw more light towards understanding the pathogenesis of colitis in IBD. These findings indicate that CCR6 could potentially serve as a diagnostic marker and a novel therapeutic target.

Chapter 1

Introduction

Inflammatory bowel disease (IBD) is an immune-mediated disorder characterised by lifelong, chronic intestinal inflammation [1-3]. Globally, IBD is highly prevalent among young adults and has a huge impact on their quality of life [4]. Australia has been reported as having the highest incidence of IBD: around 75,000 people are affected and this number is predicted to increase to 100,000 by the year 2022 [5]. In Australia, the economic burden of IBD is around 2.7 billion dollars every year. The onset of IBD occurs between the age of 5–40 in both men and women, affecting approximately 1 in 250 people [6,7]. Epidemiological studies reveal that the high incidence and prevalence of IBD is now in a stabilised pattern in continents such as America and Australia, as well as in some European countries [8,9]. Additionally, an increase in the incidence of IBD has been observed in the last decade in some developing countries of Asia and Africa [8]. Such a change in IBD incidence may be attributed to factors such as westernisation of lifestyle, diet, increased consumption of foods high in sugar, improved hygiene and increased use of antibiotics [8]. Crohn's disease (CD) and ulcerative colitis (UC) are the two most common forms of IBD affecting the gastrointestinal tract [3]. CD is a transmural, granulomatous condition commonly involving the ileum and the colon. In contrast, UC specifically involves the colon of the intestine and manifests as superficial inflammation confined to the mucosal and submucosal layers of the intestinal wall [3,10]. Overlapping symptoms of CD and UC are chronic relapsing flares associated with rectal

bleeding, abdominal pain and/or diarrhoea [10,11]. Colorectal cancer and a toxic megacolon are life-threatening gastrointestinal complications of UC and CD patients [3,12,13]. Extra-intestinal manifestations such as musculoskeletal, dermatologic, hepatopancreatobiliary, ocular, renal and pulmonary manifestations were observed in 25–40% of IBD patients [14].

1.1 Crohn's disease

CD can affect any part of the gastrointestinal tract, from the mouth to the anus, but is most commonly localised to the ileum and the colon. CD is characterised by a transmural granulomatous inflammation affecting all layers of the intestinal wall. The inflamed regions demonstrate skip lesions, the presence of deep ulceration of bowel mucosa, perianal fistulas, rectal sparing, aphthous ulcers, and thickening of the intestinal wall [15-17]. The symptoms of CD are persistent diarrhoea, abdominal pain and cramps, development of bowel strictures and discontinuous ulcers in the digestive tract. Treatment for CD is currently symptomatic with the use of aminosalicylates and immune modulators [18].

1.2 Ulcerative colitis

UC is a disease of the colon which causes inflammation confined to the mucosal and submucosal layers of the intestinal wall [17]. UC manifests as frequent bouts of bloody diarrhoea, abdominal pain and cramps, and is associated with fever and loss of appetite. The inflammation is continuous and usually involves the rectum and part of the proximal colon or the entire colon [17]. Interestingly, early appendectomy and smoking confer beneficial effects in patients suffering from UC, but not in CD [19]. There is convincing evidence proving a lower incidence of UC in smokers and presentation of a milder

disease course than in non–smokers [20]. Surgical options for UC, such as colectomy, are reserved for complications such as fulminant colitis, toxic megacolon and dysplasia/colorectal cancer [21].

1.3 Pathogenesis of IBD

The pathogenesis leading to the disease process has been widely researched, yet the detailed mechanisms remain elusive [22]. While the aetiology of IBD remains unclear, evidence suggests impaired innate and adaptive immune responses play a significant role in the initiation of IBD [4,23-25]. An altered immune response against luminal bacteria in genetically predisposed individuals is reported to be a primary risk factor for IBD (Figure 1.1) [22]. Mucosal breaches by luminal microflora initiating an immune response that can be exacerbated by an altered balance between commensal and pathogenic bacteria are considered another risk factor [26].

Environmental factors such as the use of antibiotics, smoking, westernised diet, psychological stress, appendectomy, oral contraceptives, antibiotics, atypical mycobacterial infection, episodes of childhood infections, and increased intestinal permeability can act as possible triggers for the initiation of IBD [4]. Mawdsley et al. [27] reported that psychological stress augments disease activity in IBD. In IBD patients, episodes of acute and chronic stress influence the immune mechanisms and may be relevant to the initiation of IBD pathogenesis.

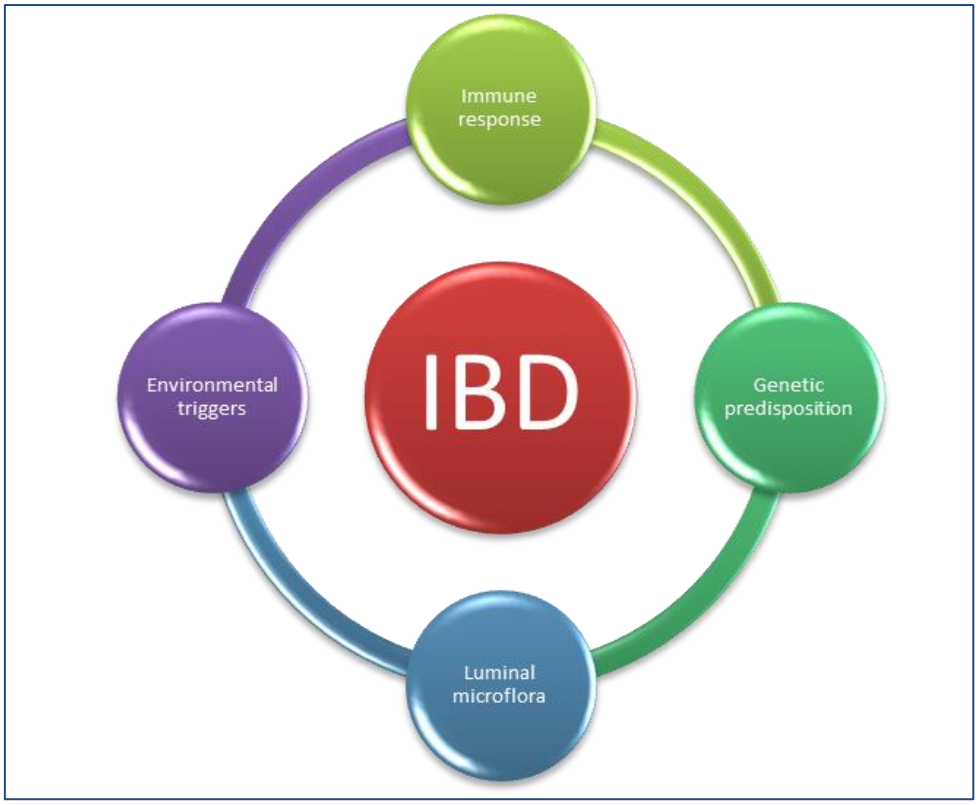


Figure 1.1: Multiple factors contributing to the initiation of IBD.
Genetic predisposition of individuals plays a major role in the initiation of IBD. Environmental factors and luminal microflora may act as triggers in the presence of a dysregulated immune response that exacerbates IBD.

Genome-wide association studies (GWAS) have subsequently identified up to 71 new associations in addition to the existing loci, bringing the total to around 163 risk-conferring loci associated with IBD [28]. Genetic susceptibility is one of the major contributing factors to the onset of IBD [29,30]. Numerous studies have identified genetic factors associated with IBD and have found that the overall expression appears to be polygenic [31]. GWAS have specifically shown new insights into the complex pathway of IBD pathogenesis [32].

The first IBD susceptibility gene, identified using candidate gene studies, was mapped to chromosome 16 and was identified as nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*), also known as caspase recruitment domain-containing protein 15 (CARD15*)* [10,31]. The identification of the gene *NOD2/CARD15* was pivotal

in providing a better understanding of IBD pathogenesis and emphasising a link between development and innate immunity [31]. The association between *NOD2* mutations and CD is much stronger than with UC [10]. Of the genes identified, some are specific to CD or UC, whereas some are shared between CD and UC (Figure 1.2) [10,28,31]. Interestingly, many of the genes associated with IBD, such as *NOD2, TLR, JAK2* and *STAT3* play a role in the innate immune response [10,31].

Dysregulation of chemokines and chemokine receptors are thought to be particularly significant in the altered mucosal immune responses in IBD. Researchers have identified chemokine and chemokine receptors as potential targets for therapy in human diseases [33,34]. The C-C chemokine receptor type 6 (*CCR6*) gene has recently been identified to be associated with Crohn's disease [35].

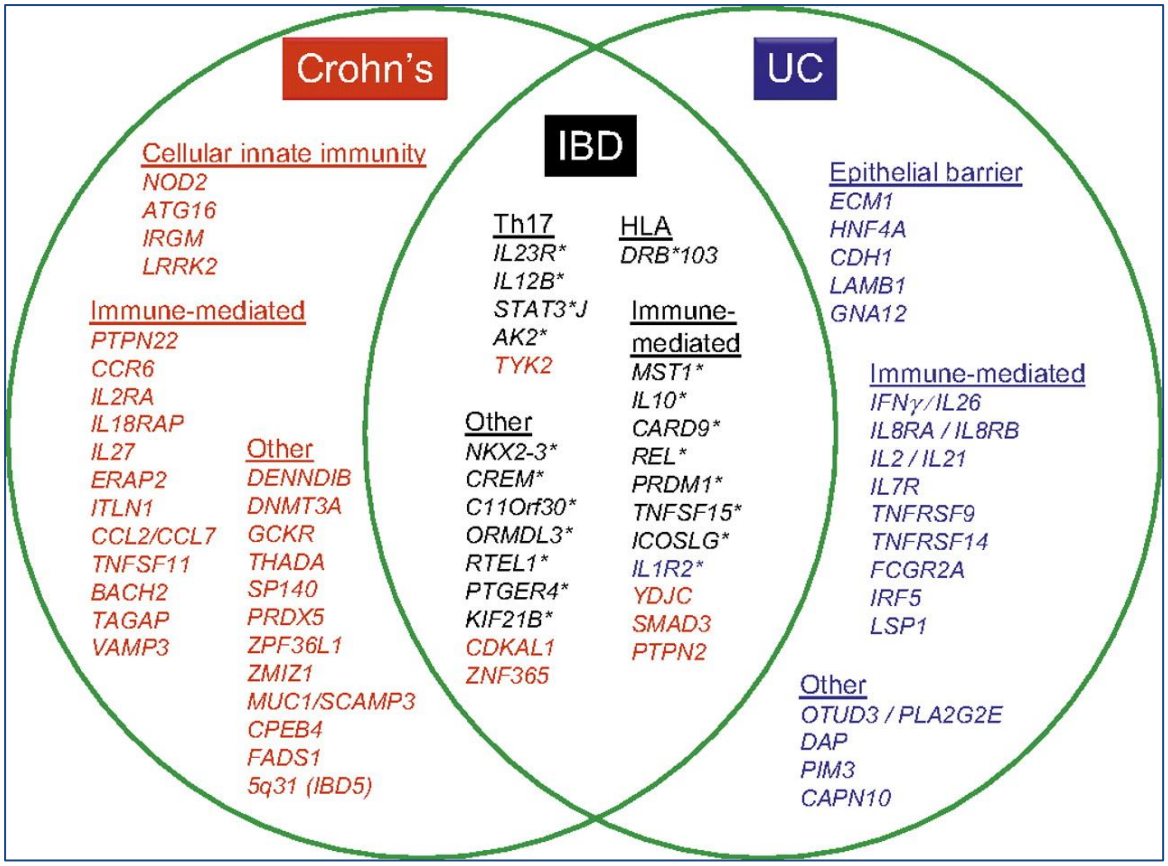


Figure 1.2: Unique and shared susceptibility gene loci in CD and ulcerative colitis. The loci, depicted by lead gene name attaining genome-wide significance, are shown for CD in red, UC in blue, and IBD in black. Reproduced from C.W. Lees et al. [35].

1.4 Intestinal immune system

The intestinal epithelium consists of a single layer of epithelial cells and is interconnected by close-knit junctions covered by mucus, providing a mucosal barrier that prevents the entry of luminal toxins, microorganisms and foreign antigens [36]. The intestinal epithelium encounters up to 10^{14} of luminal microbes [37], comprising over 500 different species, on a regular basis [38]. It has selective permeability and allows passage of dietary nutrients, water and electrolytes to enter into the circulation from the intestinal lumen [36,39,40]. The intestinal mucosa is rich in tissue macrophages and lymphocytes [41]. The prevention of bacterial penetration, invasion and systemic spread is essential in

maintaining immune homeostasis [36]. Immunological tolerance towards commensal microbes and the effective responses mounted to an external pathogen needs to be well balanced for both preventions of disease and maintenance of homeostasis [42]. Mucin, a protein produced by intestinal goblet cells, forms an integral part of the mucus layer covering the intestinal epithelium along with antibodies, defensins and lysozymes [43-45]. The outer loose mucus layer serves as the first physical and chemical barrier to the luminal microbes, while the inner mucus layer is essentially sterile and contains a number of antimicrobial peptides [41,46]. A range of antimicrobial peptides is produced by Paneth cells, which are granulated cells located at the base of small intestinal crypts. Paneth cells prevent microbial invasion in the small intestine by secreting α-defensins, lysozymes and secretory phospholipase A2 [47]. The epithelial cells are involved in the host's defence mechanism and they internalise the incoming gram-negative bacteria through phagocytosis. Goblet cells are also involved in delivering luminal antigens to the immature dendritic cells for antigen sampling [45].

Microbial cell wall components such as lipopolysaccharide (LPS) can stimulate enterocytes, provoking an innate immune response with an increased production of interleukin 8 (IL-8) via activation of the transcription factor nuclear factor kappa B (NF-κB) [48]. The microfold cell (M cell) is a unique cell type which is present in the follicle-associated epithelium (FAE) covering the Peyer's patches. These, along with intestinal dendritic cells, are involved in luminal antigen sampling [49]. Neutra et al. [50] suggested that the uptake of immune complexes by M cells may regulate mucosal immune responses, but the exact significance of the interaction between the M cells and the intestinal antibodies remains unknown.

1.4.1 T cells

The dendritic cells mature as they migrate from intestinal peripheral or inflamed tissue and transfer antigens to the mesenteric lymph node where naïve T cells undergo differentiation after an antigen encounter [51]. The differentiation of T cells often depends on the cytokine environment and an intricate association between cells of innate and adaptive immune responses [52,53]. $CD4^+$ T cells differentiate into T regulatory cells, Th1, Th2 and Th17. Both Th1 and Th2 produce some cytokines such as interferon-γ (IFN-γ), lymphotoxin-α (LTα), interleukin-4 (IL-4), IL-5 and IL-13, in response to stimuli by pathogens [54].

Until recently, Th1 cells were shown to be involved in mediating a number of autoimmune conditions [54,55]. T helper lymphocytes, requiring IL-23 for differentiation and producing IL-17, IL-17F, IL-21 and IL-22, have been identified as a new subset of T cells known as Th17 effector cells [54]. Th17 effector subpopulations have been observed to have more plasticity in phenotype [52]. Th17 cells mediate dysregulated intestinal inflammation leading to the progression of the disease but the mechanics are highly debated and remain unresolved [56]. T regulatory cells are stimulated by IL-10 secretion of the dendritic cells [57-61]. $CD4^+$ T cells expressing forkhead box P3 (FOXP3) promote tolerance to the intestinal flora, and IL-10 producing cells comprise a major portion of intestinal regulatory cells [61].

Antigenic peptides are presented by major histocompatibility complex (MHC) molecules to T cells which then initiate a cascade of events to destruct pathogens [62]. The intestinal tissue microenvironment and the local cytokine environment enhance the T cell receptor (TCR) activated $CD4^+$ T cell differentiation into IL-10 and TGF-β producing cells [63]. T regulatory cells assist in maintaining immunological homeostasis in the gut by avoiding inappropriate immune responses [61]. Effective communication between the intestinal epithelial barrier and innate and adaptive immune cells is vital in maintaining intestinal homeostasis and also in preventing any disease process (Figure 1.3) [64]. Impaired innate and adaptive immune response involving Th17/Th1 inflammatory response and increased inflammatory cytokine production is thought to initiate IBD [65].

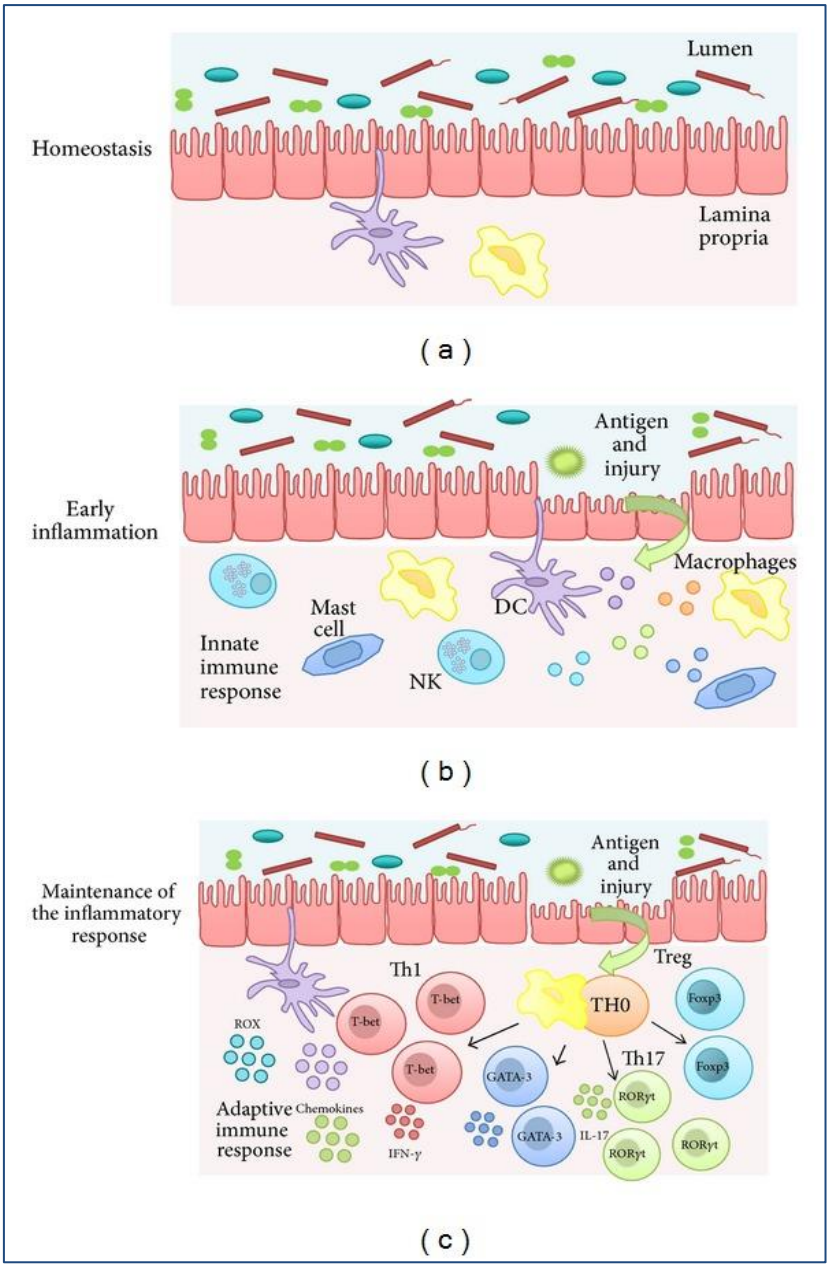


Figure 1.3: Pathophysiology of IBD.
(a) The intestinal immune system encounters numerous antigens and distinguishes between invasive organisms, dietary antigens, proteins, and commensal bacteria. The intestinal homeostasis depends on the dynamic crosstalk between the microbiota, the intestinal epithelial cells, and the resident immune cells.
(b) Various regulatory mechanisms are involved in maintaining intestinal homeostasis. The disturbance of this balance triggers the chronic inflammatory process found in inflammatory bowel disease. In early inflammation, foreign antigens activate the various innate immune cells located in the intestine, including natural killer cells, mast cells, neutrophils, macrophages, and dendritic cells.
(c) Maintenance of the inflammatory environment promotes the activation of the adaptive immune response. Aberrantly activated effector T helper (Th) cells synthesise and release different inflammatory mediators which turn into a vicious circle of inflammation leading to chronic tissue injury and epithelial damage.
Reproduced from J. Galvez [66].

1.5 Chemokines

Chemokines are a large family of small (8-12kDa) globular proteins which mediate the chemotaxis and chemo-attraction vital to the effective functioning of macrophages, lymphocytes, neutrophils and monocytes [67,68]. Chemokine activity is mediated by interaction with chemokine receptors belonging to a superfamily of seven-transmembrane-domain G-protein-coupled receptors [67]. Between 40 and 50 human chemokines have been identified so far, with approximately 20 human chemokine receptors identified, suggesting that receptors have multiple binding partners [68,69].

Chemokines are classified into two broad groups based on their functions, homeostatic and inflammatory [25,67]. Homeostatic chemokines are constitutively expressed in a particular tissue environment and are involved in physiological trafficking and cell migration [70]. Inflammatory chemokines are up-regulated and are involved in recruiting the lymphocytes, neutrophils and macrophages during an inflammatory response [67]. Chemokines are involved as pro-inflammatory agents and enhance the recruitment of lymphocytes and different types of leukocytes to the site of inflammation by binding to their G-protein coupled receptors on the surface of immune cells [71]. Hence, chemokines are powerful pro-inflammatory chemotactic cytokines, requiring stringent control over their regulatory mechanism [72].

Dysregulation of chemokines is strongly implicated in many acute and chronic inflammatory diseases such as respiratory diseases, arthritis and atherosclerosis [73]. At the focal site of inflammation, inflammatory chemokines are released, facilitating the ongoing migration of effector cells in a chronic inflammatory environment [74]. Chemokine inhibition results in an anti-inflammatory environment, whereas promoting

their activity may lead to wound-healing process. Chemokines are therefore attractive potential targets for the development of new therapeutic agents for chronic inflammatory disease [75,76].

Chemokines are classified based on the different amino acid sequence around the first two N-terminal cysteine residues [67] and divided into distinct subclasses CXC, CC, CX3C and XC, and an addition of R for the receptor, followed by a number indicating the order of discovery [69,77,78]. Chemokines have three β- pleated sheets and a carbon terminal α-helix with disulphide bonds connecting cysteine residues [79]. Chemokines consist of structurally related secreted proteins of 67–127 amino acids in length [78] and they exist as a monomer, dimer or tetramer, the functional form being a monomer. The amino terminus NH_2 is the most critical binding and activation site for the receptor-ligand (Figure 1.4), and the secondary binding site is present in the flexible loop region that is followed by the second cysteine [74]. Chemokines preferentially bind to seven-transmembrane G-protein coupled receptors which are found on T cells and B cells and also on other cells such as neurons and endothelial cells [68]. Many chemokines bind to multiple receptors (Figure 1.5), whereas some chemokines bind specifically to a single receptor [80].

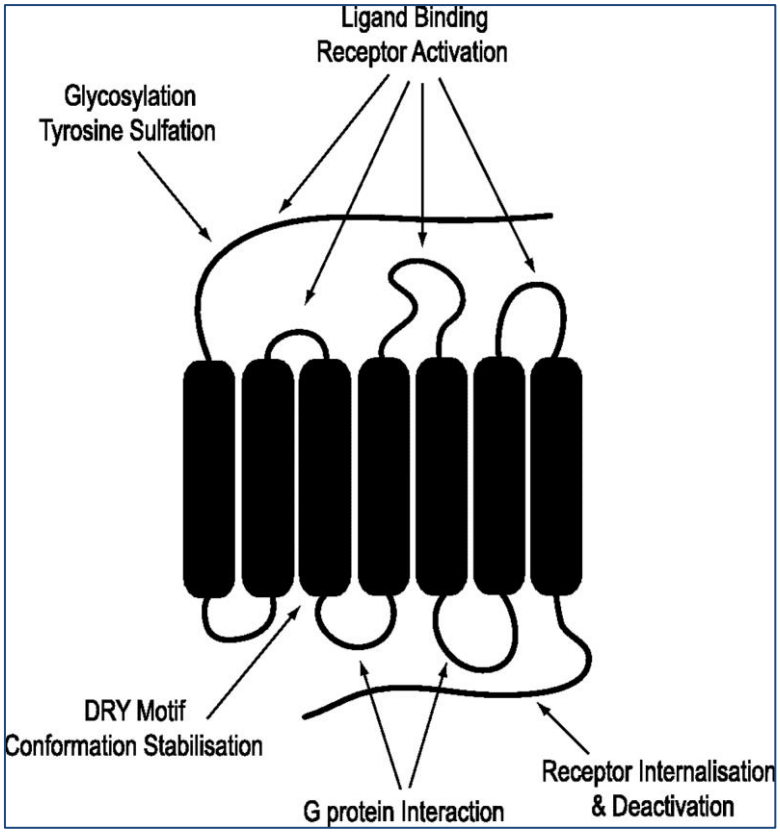


Figure 1.4: Structure and functional regions of chemokine receptors.
Structure of seven-transmembrane G-protein coupled receptors and binding sites of chemokine receptors for ligand binding, receptor activation and internalisation. Reproduced from G.E. White, A.J. Iqbal & D.R. Greaves [81].

Table 1.1: Nomenclature of Chemokine receptors and ligands.

Classification	Receptors	Systematic names of Ligands	Human Ligands	Mouse Ligands	Function
C family	XCR1	XCL1, XCL2	Lymphotactin SCM-1β	Lymphotactin Unknown	Inflammatory
CX3C family	CX3CR1	CX3CL1	Fractalkine	Fractalkine	Inflammatory
CC family	CCR1	CCL3	MIP-1α/LD78α	MIP-1α	Inflammatory
		CCL5	RANTES	RANTES	
		CCL 7	MCP-3	MARC	
		CCL14	HCC-1	Unknown	
		CCL15	HCC-2	Unknown	
		CCL16	HCC-4	Unknown	
		CCL23	MPIF-1	CCL6, C10	
	CCR2	CCL2	MCP-1/MCAF	JE	Inflammatory
		CCL7	MCP-3	MARC	
		CCL8	MCP-2	MCP-2	
		CCL13	MCP-4	Unknown	
		CCL16	HCC-4/LEC	LCC-1	
	CCR3	CCL5	RANTES	RANTES	Inflammatory
		CCL7	MCP-3	MARC	
		CCL 8	MCP-2	MCP-2	

		CCL11	Eotaxin	Eotaxin	
		CCL13	MCP-4	Unknown	
		CCL15	HCC-2/Lkn1/MIP-1δ	Unknown	
		CCL16	HCC-4/LEC	LCC-1	
		CCL24	Eotaxin-2	Unknown	
		CCL26	Eotaxin-3	Unknown	
		CCL28	MEC	ALP/CTACK	
	CCR4	CCL17, CCL22	TARC, MDC	TARC,ABCD-1	Homeostatic
	CCR5	CCL3	MIP-1α/LD78α	MIP-1α	Inflammatory
		CCL4	MIP-1β	MIP-1β	
		CCL5	RANTES	RANTES	
		CCL8	MCP-2	MCP-2	
		CCL11	Eotaxin	Eotaxin	
		CCL14	HCC-1	Unknown	
		CCL16	HCC-4/LEC	LCC-1	
	CCR6	CCL20	MIP3-α	MIP3-α	Homeostatic
	CCR7	CCL19 CCL21	MIP-3β,ELC 6Ckine, SLC	MIP-3β 6Ckine, SLC	Homeostatic
	CCR8	CCL1	I-309	TCA-3, P500	Homeostatic

	CCR9	CCL25	TECK	MPIF-2	Homeostatic
	CCR10	CCL27, CCL28	CTACK/ILC, MEC	CTACK/ILC, ALP/CTACK	Homeostatic
CXC family	CXCR1	CXCL6, CXCL 7, CXCL 8	GCP-2, NAP-2, IL-8	GCP-2/LIX, Unknown, Unknown	Inflammatory
	CXCR2	CXCL1 CXCL 2 CXCL 3	GROα/MGSAα GROβ/MGSAβ GROγ/MGSAγ	GRO/KC GRO/KC GRO/KC	Inflammatory
	CXCR3-A	CXCL9, CXCL 10, CXCL 11	MIG,IP-10, I-TAC	MIG,IP-10 I-TAC	Inflammatory
	CXCR3-B	CXCL4, 9, 10 &11	PF4,MIG,IP-10, I- TAC	PF4, MIG,IP-10,I- TAC	Inflammatory
	CXCR4	CXCL12	SDF-1α/β	SDF-1	Homeostatic
	CXCR5	CXCL13	BCA-1	BLC	Homeostatic
	CXCR6	CXCL16	Unknown	Unknown	Inflammatory
	CXCR7	CXCL12	SDF-1α/β	SDF-1	Inflammatory

Adapted from [67,80,82-84].

1.5.1 Chemokine ligand CCL20

The chemokine receptor CCR6 uniquely interacts with the chemokine ligand CCL20 (also known as Exodus-1), the macrophage inflammatory protein (MIP-3α), and the liver activation-regulated chemokine (LARC) [85-87]. Hieshima et al. [88] were the first to identify CCL20, a novel chemokine ligand expressed in the normal liver. The *Ccl20* gene is located on chromosome 2q33-37 [88,89]. The chemokine CCL20 is largely expressed in the lung, liver and appendix, and to a lesser extent in the thymus and intestine. It is also expressed in peripheral blood lymphocytes and is up-regulated during an inflammatory stimulus [85]. Furthermore, the expression of *Ccl20* is also found in various types of epithelial cells such as pulmonary epithelial cells, keratinocytes and also in intestinal epithelial cells. *Ccl20* reportedly is constitutively expressed by B cells, dendritic cells and neutrophils, and its expression can be stimulated using pro-inflammatory ligands [90]. Follicle-associated epithelial cells (FAE) covering the Peyer's patches of the intestine and the isolated lymphoid follicles (ILFs) are involved in the synthesis of Ccl20 [25]. *Ccl20* was reported to be expressed mainly in Th17 cells and not expressed by either Treg cells or T helper subsets [91]. Up-regulation of *Ccl20* expression in intestinal epithelial cells can be seen in response to induction by invasive or non-invasive flagellated bacteria, suggesting a mechanistic link between Ccl20 synthesis and TLR3 [90,92].

Furthermore, pro-inflammatory cytokines such as IL-1α or tumour necrosis factor (TNF) produced during acute intestinal inflammation also stimulate and up-regulate *Ccl20* expression [93]. Brand et al. reported that in the skin fibroblasts of chronic obstructive pulmonary disorder (COPD) patients, the *Ccl20* expression is driven by IL-1β and αvβ8-mediated TGF-β activation [94,95]. The periportal expression of *Ccl20* in the liver facilitates CCR6+ve cells being recruited to encounter incoming invading micro-

organisms, however, the periportal site is also the primary entry for colorectal cancer cells [86,88,96]. The ubiquitous presence of *Ccl20* in inflamed mucosal sites suggests its possible role in the initiation and recruitment of adaptive immune responses.

1.5.2 Chemokine receptor CCR6

Chemokine receptor 6, originally known as STRL22, was identified in 1997 by Liao et al. [97]. The *CCR6* gene in humans is located on chromosome 6q27 and is expressed in the intestine by dendritic cells (DC), subsets of T cells ($CD4^+$, $CD8^+$), and in most B cells [67,98-100]. Furthermore, *CCR6* is also expressed by central and effector memory T cells which are characterised by the expression of CCR7 [101,102]. Apart from the expression of CCR6 on Th17 cells, CCR6 recently was reported as being expressed by IL-22-producing NK cells, IL-17 producing γ/δ T cells and a subpopulation of $CD4^+$ $FOXP3^+$ regulatory T (Treg) cells [103-106]. To date, *CCR6* appears to be expressed predominantly in mucosal tissues of skin and intestine [107,108]. The $CD4^+$ T cells and dendritic cells migrate into the gut mucosal tissue in response to inflammation and their migration is thought to be mediated by CCR6 [98].

Recent studies have identified CCR6 as a specific marker for Treg cells and Th17 cells [103,109]. Th17 cells are reported to be involved in mucosal immune responses elicited due to bacterial and fungal pathogens [107]. CCR6 is expressed by both Th17 and T regulatory cells, and recent studies suggest that both have opposing roles in autoimmune diseases [91]. *Ccr6* is a susceptibility gene, strongly involved in Crohn's disease [110,111]. An *in vitro* study of differentiated T effector and T helper subsets by Yamazaki et al., [91] showed mRNA and protein expression of *Ccr6* as being very high in Th17 cells when compared with the small amounts of expression seen in Th1 and Th2

cells. Evidence of CCR6 expression in Treg cells, both *in vivo* and *in vitro*, was reported by Kleinewietfeld et al., which is consistent with the findings of Yamazaki et al. [91,103].

1.5.3 CCR6-CCL20 axis

The CCR6-CCL20 axis is an important factor in intestinal immunity. Ito et al. [75] describe the involvement of the CCR6-CCL20 axis during the normal development of innate immunity and immune homeostasis. In intestinal immune responses, especially in the innate immune response where CCR6-mediated signals are important, the CCR6-CCL20 axis might have a very significant impact on tissue damage and injury [98]. It has been suggested that CCR6 is associated with the regulation of leucocyte migration followed by effector function of T cells, all of which are considered to be important factors in gut immunity [98,110]. Induction of CCL20 by bacterial lipopolysaccharide (LPS) endotoxin in response to infection occurring in intestinal epithelial cells has been demonstrated [112]. The CCR6-CCL20 axis also mediates the chemotaxis of dendritic cells and macrophages by facilitating their migration to the site of inflammation [98].

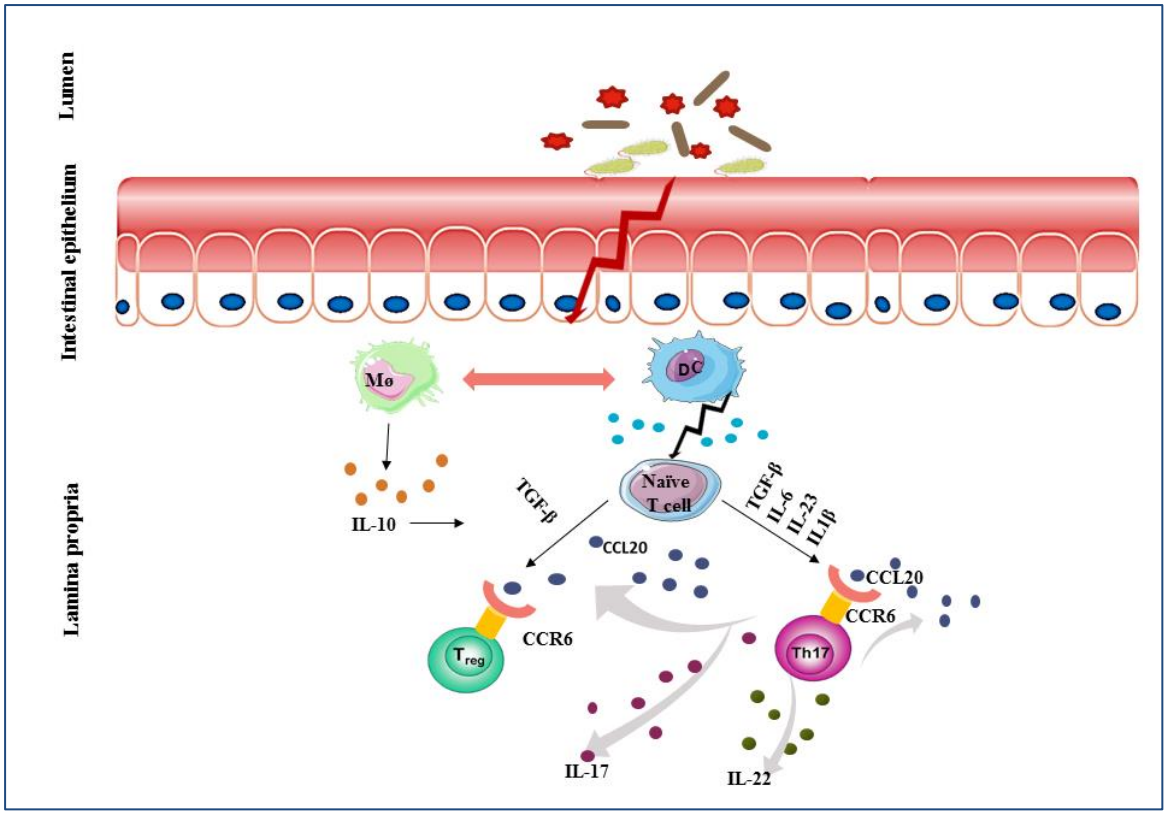


Figure 1.5: Schematic representation of CCR6 role in intestinal inflammation.
CCR6 is expressed in both T regulatory cells and Th17 cells thought to be the pivotal link between anti-inflammatory and pro-inflammatory cells. During inflammatory stimulus, the CCR6-CCL20 axis is critical in skewing towards either tolerance or inflammation. (Mø- macrophages and DC- dendritic cells**)**

Le Borgne et al. [113] demonstrated the significant involvement of CCR6-CCL20 in the migration of dendritic cells from the blood to the lamina propria of the intestine during the inflammatory process (Figure 1.6) [113]. Th17 cells and Treg cells are present in large numbers in the lamina propria during normal as well as inflammatory conditions, suggesting that the axis is critical for the outcome of intestinal pathology [114]. Maintaining the balance between inflammatory cells and T regulatory cells is vital for the outcome of intestinal pathologies [114]. The CCR6-CCL20 axis has been demonstrated to play an important role in many lung and gut disorders [98]. Furthermore, the CCR6-CCL20 axis is of notable importance in colorectal cancer and in autoimmune diseases

[103,115]. The CCR6-CCL20 axis appears to hold promise as the new target for therapeutic interventions in many diseases, including IBD [114].

CCL20 was reported to be expressed only in Th17 cells and it was not expressed by either Treg cells or T helper subsets [91]. In intestinal immune responses, especially in the innate immune response where CCR6-mediated signals are important, it might have a very significant impact on tissue damage and injury. Frick et al. [96] emphasise the need for more functional studies to prove the role of the CCR6-CCL20 axis in tumour development. Recent reports from Uchida et al. [116] suggested that the CCR6-CCL20 axis plays a significant role in ensuing pathological changes in paediatric ulcerative colitis patients when compared to the adult patients. Uchida et al. [116] demonstrated a significant increase in the production of *Ccl20* by colonic epithelial cells and increased expression of *Ccr*6 in the infiltrating inflammatory cells of the mucosal layer. The statistically significant findings corresponded to the histological outcome, leading to an increase in the disease severity being observed in paediatric patients compared to adult patients.

Studies using *Ccr6*$^{-/-}$ mice suggest that the CCR6-CCL20 axis has an important role in the immune process leading to inflammatory bowel disease [87]. Wild-type Th17 cells or *Ccr6*$^{-/-}$ T cells, when transferred into a *Rag1*$^{-/-}$ severe combined immune-deficient (SCID) mouse, resulted in severe intestinal inflammation [104,117]. Reduction in the population of Th17 and Treg cells was noted in this process [104,117]. According to these results, Lee et al. [117] suggest that the function of CCR6 is more important to Treg cells than Th17 cells. The relationship between CCL20 and its receptor CCR6 determines the balance between Treg and Th17. The outcome largely relies on the selective skewing of CCR6-CCL20 to establish whether the prominence of either Treg or Th17 is an essential

factor in the process of intestinal inflammation. The responses towards CCL20 by both Th17 and Treg cells which also express CCR6 leads to a feedback loop resulting in intestinal inflammation.

1.6 Mouse models of IBD

Mouse models have been used in many areas of biomedical research since they bear a very close similarity physiologically to humans. Researchers use mouse models to get a better understanding of the disease process and to develop and trial new therapeutics [118,119] and, as such, they are regarded as a valuable tool in IBD [120]. Based on the development of colitis, mouse models can be broadly categorised as:

1. Chemically induced colitis

2. Adoptive T cell transfer induced colitis

3. Genetically engineered models of colitis

4. Spontaneous models of colitis

1.6.1 Chemically-induced Colitis

The commonly used chemicals to induce colitis are dextran sodium sulphate (DSS), a chemical colitogenic, oxazolone and 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). The administration of chemicals such as DSS causes the animals to develop inflammation resembling human colitis [121-123]. DSS is the most widely used chemical to induce colitis in mouse models as it is easy to administer: it is dissolved in drinking water in single or multiple cycles and leads to acute or chronic colitis [124]. The cytotoxic effect of DSS is conferred onto the intestinal epithelial cells, efficiently breaching the barrier

function and exposing the luminal microbial flora of the bowel [124,125]. The colitis induced by oxazolone and DSS, when histologically analysed, seem to show close similarity to UC, whereas TNBS-induced colitis shows similarity to CD [126]. Induction of intestinal perforation causing the death of animals, diverse intestinal microflora, and optimisation of dosages owing to the genetic heterogeneity of mice are thought to be the disadvantages of the chemically induced mouse models [121].

1.6.2 Adoptive T cell transfer induced colitis

The adoptive transfer model provides extensive knowledge of the role of T cells, B cells and innate cells in various stages of inflammatory colitis in mice [127-129]. Transfer of $CD45RB^{high}$ naïve T lymphocytes occurs into severe combined immune-deficient (SCID) or recombination activation gene 1 ($Rag1^{-/-}$) immune-deficient recipients as these are devoid of T regulatory cells (Treg) cells. The colitis demonstrated in adoptive transfer mouse models closely mimics chronic inflammation as seen in human IBD patients. The severity of the inflammation varies, largely depending on the choice of the donor strain and the recipient strain [130,131]. As a result of the interaction between naïve donor T cells and the innate immune cells of the recipient, chronic inflammation ensues. Treatment with antibiotics and anti-inflammatory drugs ameliorates adoptive transfer colitis similar to IBD [130,132]. Lack of Treg cells in the adoptive transfer model leads to intolerance towards luminal microflora and an abnormal immune response is elicited. The altered immune response, one of the contributing factors for the onset of IBD, is well demonstrated by exhibiting poor immune homeostasis in adoptive transfer colitis. The disadvantages of using mouse models are that they are artificial and the unnaturally

created immune environment in immune-deficient mice is in contrast to the naturally occurring human IBD environment [130,132].

1.6.3 Genetically engineered mouse models of colitis

Genetically engineered mouse models are the most widely used and around 74 spontaneous mouse models of IBD are classified under this category [133]. These genetically engineered mouse models are recent advances and arise from interventions such as gene insertions/gene alterations or gene deletions in known loci of interest, resulting in alteration of the immune mechanism, and they are used to study adaptive and innate immune functions [134]. Cell-specific conditional knockout, conditional transgenic, knockin, conventional gene knockout, transgenic, conventional transgenic, mutation knockin, and N-ethyl-N-nitrosourea (ENU) mutagenesis are all among these [135,136].

1.6.3.1 Ccr6$^{-/-}$ mice

Ccr6$^{-/-}$ mice were developed by replacing the mouse *Ccr6* gene by homologous recombination technique and targeted disruption of the gene [137]. *Ccr6*$^{-/-}$ mouse models are used to understand the immune responses and the role of the chemokine axis CCR6-CCL20 in the intestine [87]. CCR6 is found to be expressed selectively by the T cells, B cells and also in the subpopulations of dendritic cells of mice and humans [100,138]. The chemokine ligand CCL20 has its specific chemokine receptor CCR6 [86], only found to interact with mice and humans [87].

Ccr6$^{-/-}$ mice possess underdeveloped Peyer's patches and, as a result, reduction of CCL20 expression by FAE was reported along with a compromised development of isolated

lymphoid follicles having been noted in *Ccr6*$^{-/-}$ mice [25]. These mice show a decrease of CD11b^{+}, CD11c^{+} myeloid dendritic cells in the subepithelial dome of Peyer's patches. These are displaced into the inter-follicular region and may not exist in Peyer's patches of *Ccr6*$^{-/-}$ mice [137,139].

The *Ccr6*$^{-/-}$ mice have an impaired humoral response to orally administered antigen and they also fail to respond to rotavirus, an enteric pathogen as demonstrated by Cook et al.[140]. The developmental defects of mucosal-inductive places in *Ccr6*$^{-/-}$ mice are the reason behind decreased immunoglobulin A (IgA) production to orally administered antigens [25]. *Ccr6*$^{-/-}$ mice are useful in studying the function of M cells, as the interaction of the chemokine axis CCR6-CCL20 is said to play an important role in M cell differentiation [141]. Studies using *Ccr6*$^{-/-}$ mice also show an increase in TCRα/β and T cell subpopulations which indicates a possible role of this chemokine receptor in immune regulation [95].

In contrast, the *Ccr6*$^{-/-}$ mice confer normal systemic immune responses to subcutaneous antigens. Research suggested CCR6 to be a regulator of both humoral and lymphocyte homeostasis in the intestine [139]. *Ccr6*$^{-/-}$ mice, when treated with DSS, developed less severe intestinal inflammation compared to wild-type mice [87]. Interestingly, when *Ccr6*$^{-/-}$ mice were treated with TNBS they showed increased susceptibility to intestinal inflammation in otherwise genetically resistant strains [87,114,142].

Studies using *Ccr6*$^{-/-}$ mice suggest that the CCR6-CCL20 axis plays an important role in the immune process leading to inflammatory bowel disease [87]. *Ccr6*$^{-/-}$ mice have an increased T cell subpopulation within the mucosal layer of the intestine [137,140], with CCR6 being expressed on the cell surface of Th17 cells [143]. Interestingly, not all mouse

Th17 cells expresses CCR6, unlike in humans where all Th17 cells are shown to express CCR6 [91].

It was observed that the alterations of the gut leukocyte homeostasis and the cytokine environment might confer disrupting effects on the functionality of the immune system [87,140]. The developmental defect of mucosal-associated lymphoid tissues (MALT), which are inductive places in *Ccr6*$^{-/-}$ mice, is due to decreased IgA production to orally administered antigens [25]. In contrast, the *Ccr6*$^{-/-}$ mice display normal systemic immune responses to subcutaneous antigens [140]. Research suggested CCR6 to be a regulator of both humoral and lymphocyte homeostasis in the intestine [140]. *Ccr6*$^{-/-}$ mice were used to demonstrate the role of CCR6 and its importance in many lung and gut disorders [98].

1.6.4 Spontaneous models of colitis

1.6.4.1 Winnie mice

In the field of IBD study, there are two valuable mouse models derived from ENU mutagenesis targeting the mucin 2 gene (*Muc2*), namely Winnie and Eeyore. A point mutation in Muc2 causes misfolding of the mucin and initiates an unfolded-protein response (UPR), resulting in endoplasmic reticulum (ER) stress [43]. Initiation of chronic inflammation involving both innate and adaptive immunity is mediated by ER stress and leads to the development of severe colitis spontaneously in mice [132]. Winnie is one such mouse model which is proven to be a spontaneous model of intestinal inflammation [43]. One significant feature of this model is that Winnie mice have a primary epithelial defect but their underlying immune system is normal. The nature of the inflammatory response in Winnie mice has been extensively characterised (Figure 1.7) [43,144,145].

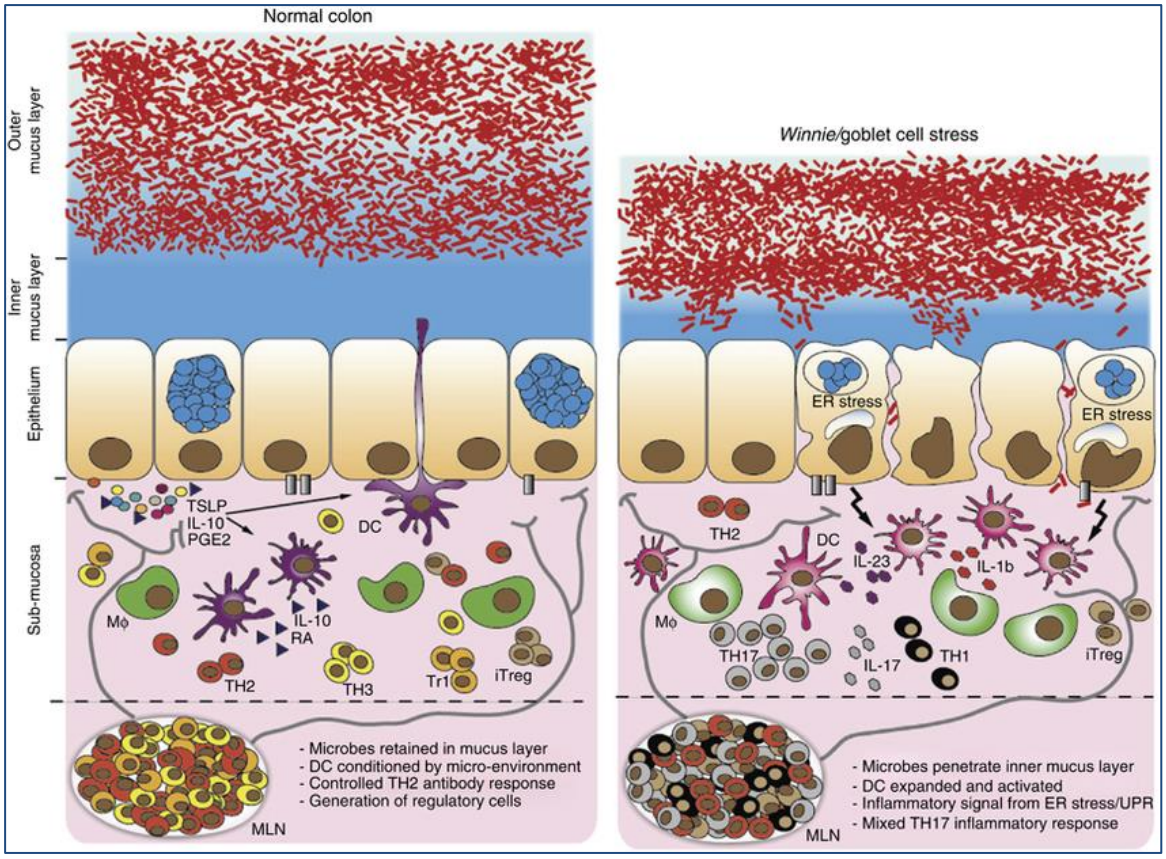


Figure 1.6: Schematic representation of the initiation of inflammation in Winnie mice.
Reproduced from R.D. Eri et al. [145].

There is also an increase in gut permeability leading to the occurrence of severe colitis [43]. Increased production of inflammatory cytokines such as TNF-α, IL-1β and IFN-γ is seen in the distal colon explant cultures of Winnie mice. Also notably, an increased expression of Th17-related genes *Il-17a, Il-17f, Tgf-β1* and *Ccr6* in the intestinal mucosa, in particular, is seen in the distal colon of Winnie mice [43]. The Winnie mice display Th1, Th2 and dominant IL-23/Th17 immune responses similar to the human IBD patients. The pathogenic mechanisms in Winnie mice closely resemble ulcerative colitis, making these mice a valuable tool in IBD research [43,132].

1.6.4.2 SAMP1/Yit mice

The SAMP1/Yit mouse model was originally derived from the senescence-accelerated mouse P series (SAMP1) and was generated by Takeda et al. [146]. The SAMP1/Yit strain was utilised to study the genetics of ageing and accelerated senescence in a short-lived strain. The SAMP1/Yit strain develops spontaneous inflammation with a primary epithelial cell defect and shows a close resemblance to Crohn's disease pathology [147-149]. The discontinuous pattern of inflammatory lesions was mainly observed in the terminal ileum and slightly less so in the caecum [149]. Interestingly, Matsumoto et al. [147] identified that under gnotobiotic conditions the SAMP1/Yit mouse strain was free from intestinal inflammation.

1.6.4.3 TLR4 $^{-/-}$ mice

Toll-like receptor 4 is a protein encoded by the TLR4 gene. The *TLR4*$^{-/-}$ mouse model has a point mutation in the coding region which provides insight into the role of these receptors in the pathogenesis of inflammatory bowel disease [150-152]. The TLR4 point mutation mouse model was first developed by Hoshino et al. [153]. A few TLR family members serve as a cell-surface receptor for lipopolysaccharide (LPS), the major cell-wall component of Gram-negative bacteria [154].

However, *TLR4*$^{-/-}$ mice are unresponsive to LPS as TLR4 is integral to the LPS response, which assists in better understanding the innate immune responses to bacteria and their role in this model [154,155].

1.7 Therapeutic strategies for IBD

Conventional therapies involve the use of anti-inflammatory drugs: aminosalicylates and steroids. Immunomodulators such as azathioprine, 6-mercaptopurine, methotrexate or cyclosporine are also being used in patients who are resistant to or dependant on steroids. Biological therapies have gained importance in IBD therapy and targeted therapies with anti-inflammatory cytokine-therapy such as an anti-TNF-α antibody, anti-IL-6R, and anti-IL-12 or toxin-conjugated anti IL-7R, recombinant cytokines (IL-10 or IL-11) have been used for IBD therapy [18]. Current therapeutic advances in the clinical management of patients with IBD include biological agents such as TNF blockers, and inhibitors of cytokines such as IL-6, IL-6R, IL-12 and IL-23 blockers. Other therapeutic strategies such as modulators of cytokine signalling events including JAK inhibitors or SMAD7 blockers and inhibitors of transcription factors GATA3 or RORγt are currently being investigated in clinical trials [156].

The CCR6-CCL20 axis has been considered as a therapeutic target for autoimmune diseases such as psoriasis, dry eye disease (DED), rheumatoid arthritis (RA) and experimental autoimmune encephalomyelitis (EAE) [157-160]. Chemokine targeted therapies have lately gained interest as they are considered to be a specialised form of treatment. The targeting of chemokines is likely to be an alternative to existing conventional treatments and biological treatments such as the use of TNF blockers along with disease-modifying drugs which result in immunosuppression as one of the side-effects among many others in some of the recipients [160].

1.8 Summary

As demonstrated by evidence from various research groups, the CCR6-CCL20 axis may be a pivotal link in the balance between the Th17 cells and T regulatory cells [161]. The specific role of this axis is unknown. In this study, the overall aim is to develop a *CCR6*-deficient spontaneous mouse model and to understand the role of the CCR6-CCL20 axis in the intestinal inflammatory environment. The focus of this study is the assessment of the CCR6 role in Winnie colitis. Based on the experimental outcomes achieved in the new mouse model, further experiments involving IBD patients could be planned. If the pathogenic role of CCR6-CCL20 in Winnie colitis can be demonstrated, then future studies targeting the CCR6-CCL20 axis may be a novel approach to a plausible IBD treatment.

Research involving the CCR6-CCL20 axis and its role in various autoimmune disorders is of particular interest to the global scientific community. The CCR6-CCL20 axis needs to be further investigated as a clear understanding of its role with regard to inflammatory bowel disease is essential. CCR6 appears to be a pivotal link between the anti-inflammatory and pro-inflammatory cells, with the CCR6-CCL20 chemokine axis mediating the inflammatory pathway. The significance of this current study is to study the effects of *CCR6* deficiency in a spontaneously occurring colitis mouse model. The highlight of our model is that it is a spontaneous occurring colitis due to an SNP, unlike the existing contrived IBD mouse models. CCR6 is suggested to be involved in both immune regulation and enhancement of inflammation due to its presence in both Treg cells and Th17 cells. The interaction between the chemokine receptor and ligand in events, activation and resolution of colitis require further definition to understand the

pathological skewing toward chronic inflammation. Identification of the CCR6 role in colitis would be a valuable biomarker and hence a potential therapeutic target for IBD.

1.9 Hypothesis

Genetic ablation of the *Ccr6* gene exacerbates intestinal inflammation in a spontaneous colitis mouse model.

1.10 Project Aims

The overall aim of this study is to understand the functional role of CCR6 in colitis by developing a Winnie x *Ccr6*$^{-/-}$ mouse model.

1. Generation of Winnie x *Ccr6*$^{-/-}$ mouse model.
2. Assess the role of *Ccr6* in intestinal inflammation using clinical and histopathological parameters in Winnie x *Ccr6*$^{-/-}$ mice.
3. Analysis of immunological, molecular and cytokine profile of Winnie x *Ccr6*$^{-/-}$ mice.

Chapter 2

Materials and Methods

2.1 Experimental Mice

The mice used for this research project were imported from Jackson Immuno Research Laboratories, Inc. PA, USA, and bred directly at the University of Tasmania Cambridge Farm facility, or sourced from Monash University, Victoria. The mice were rederived and bred at a specific pathogen-free facility at Cambridge Farm and then transported to the PC2 animal facility at the Menzies Institute for Medical Research, Hobart. Mice utilised for this project (Table 2.1) were all aged between 8–12 weeks. All animal procedures were approved by the Animal Ethics Committee of the University of Tasmania (**Ethics no. A0013691**), and were conducted in accordance with the Australian code for the care and use of animals for scientific purposes 8th edition (2013), set out by the National Health and Medical Research Council.

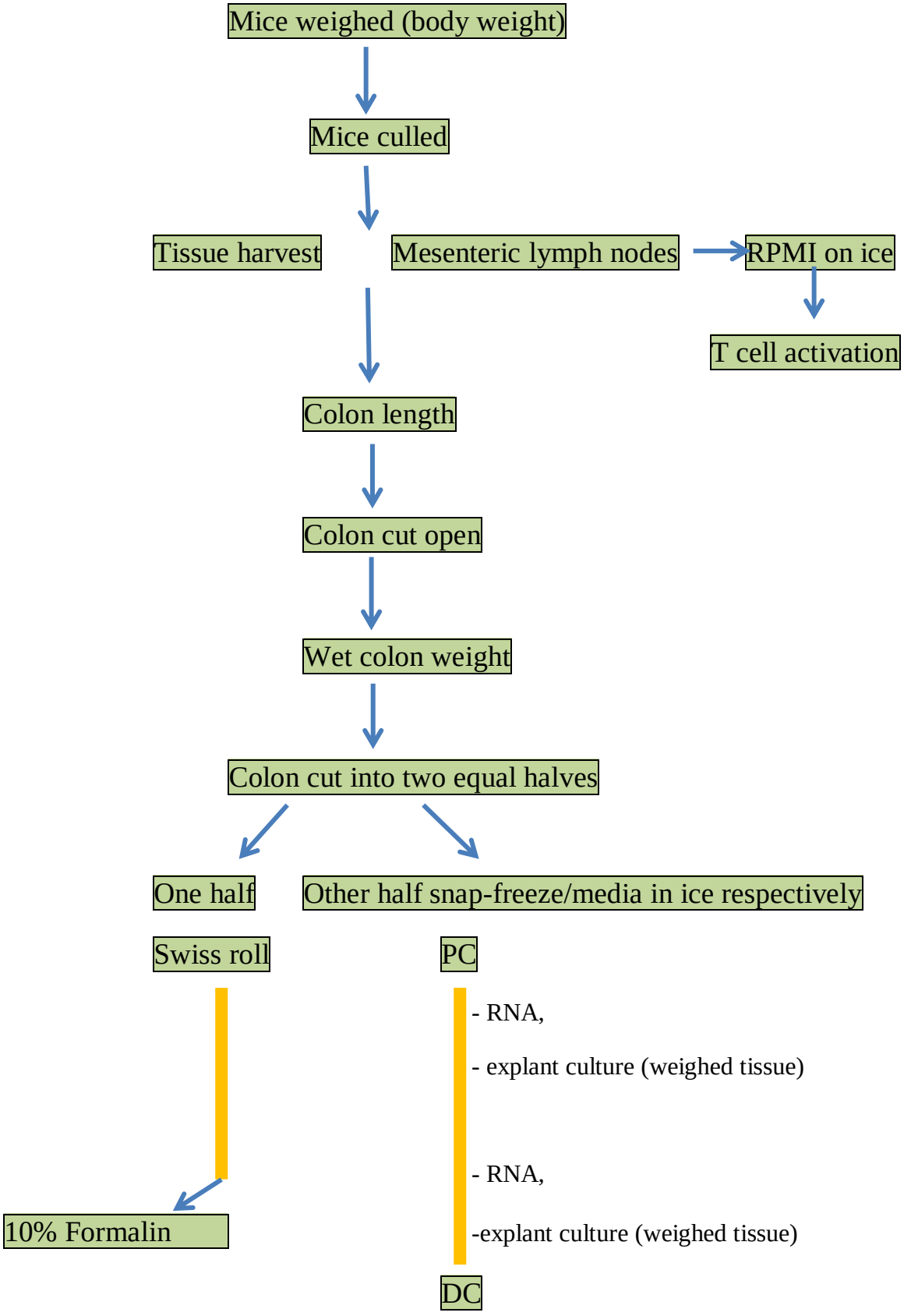
Table 2.1: Experimental strains of mice

Animal strain	Age	Sex	Phenotype
C57BL/6J (JAX® stock no-000664) Wild-type /B6	8–12 weeks	F/M	Wild type
***Ccr6*$^{-/-}$** *(JAX® stock no-005793)* B6.129P2-*Ccr6*^{tm1Dgen}/J	8–12 weeks	F/M	Targeted knockout
Winnie	8–12 weeks	F/M	Muc2 mutation
Winnie x *Ccr6*$^{-/-}$	8–12 weeks	F/M	Muc2 mutant and targeted knockout for CCR6

2.2 Clinical assessment

Mice were weighed prior to euthanasia and culled using a CO_2 chamber. Under sterile conditions, spleen and mesenteric lymph nodes were harvested. The harvested lymph nodes and spleen were stored in RPMI 1640 culture medium (Life Technologies Pty Ltd, Victoria, Australia) supplemented with 10% v/v foetal calf serum (FCS) (Life Technologies, Victoria, Australia), penicillin (100 mU/L) and streptomycin (100 mg/L) (Sigma-Aldrich Pty Ltd, NSW, Australia). The large intestine was retrieved and was placed on a non-absorbent surface. The length of the colon was measured from the caeco-colic junction to the rectum and was recorded. The whole colon was cut open in a longitudinal axis; faecal contents were removed and weighed. The opened colon was then bisected longitudinally. One half, containing the proximal and distal colon, was carefully rolled into a Swiss roll and preserved in 10% formalin until further processing. The other half of the proximal and distal colon sections were used for explant cultures. The remaining part of the colon sections was cut into pieces and snap-frozen at−80°C for RNA extraction.

Tissue Harvest from Mice



2.3 Genotyping

The DNeasy Blood & Tissue Kit (Qiagen Pty Ltd, Victoria, Australia) was used for genomic DNA (gDNA) extraction. Mouse-ear clips were lysed overnight using the supplied proteinase K and lysis buffer. Lysis was performed at 56°C with constant agitation at 500rpm using an Eppendorf ThermoMixer® C. Lysed samples were then transferred to spin columns and processed according to the manufacturer's protocol and genomic DNA eluted (200µL). DNA concentration (10ng/mL) was determined using Qubit®2.0 Fluorometer (Life Technologies, Victoria, Australia).

2.3.1 Winnie SNP genotyping assay

The presence of the Winnie Muc2 mutation was confirmed using a custom-designed Winnie SNP PCR assay (Life Technologies, Assay ID: AHCSX8U, Catalogue No: 4332077, Size: 1500rxn, 40x) diluted in 1 x TE buffer to 20x working stock. TaqMan genotyping master mix (Catalogue no: 4371355, 10mL and Catalogue no: 4371353, 1mL) or TaqMan® GTXpress™ Master Mix (Catalogue No: 4401892, 10mL) was used for the SNP assay. A volume of 5µl of 2 x Master mix, 0.5µl of 20x SNP, 3.5µl molecular grade H_2O, and 1µl of genomic DNA (10ng/µl) was used in a 10µl reaction volume for the SNP assay.

2.3.2 *Ccr6* genotyping polymerase chain reaction

Deletion of the *Ccr6* gene was confirmed using the CCR6 primers (20µM) (Table 2.2) purchased from GeneWorks Pty Ltd, Adelaide, Australia. HotStar Taq Master Mix Kit (Catalogue no: 203445, Qiagen Pty Ltd, Victoria, Australia) was used with 2µl of

genomic DNA in a reaction volume of 20µl and thermal cycling performed using a PTC-200 Thermal Cycler (MJ Research).

Table 2.2: CCR6 genotyping primer list

Primer	Sequence	Length	Tm
CCR6 5' ORF	5'---GTCATCACCACCATAATGTTG	21	55
CCR6 5' UTR	5'---TCTGCACTAGTGAGAGTGTG	20	55
CCR6 neo rev	5'---GAACGAGATCAGCAGCCTCTGTTCC	25	55

2.3.3 CCR6 gel electrophoresis

The PCR products were analysed on 2% w/v agarose gel prepared in 1X TBE buffer. A volume of 3µL of 10,000X SYBR Safe™ DNA Gel Stain (Invitrogen Technologies) was added to the agarose gel and mixed well. A volume of 5µl of DNA Easy ladder I (Bioline, NSW, Australia, Catalogue No: BIO-33046) was dispensed into the ladder well and 4µl of PCR product and 1µl of DNA loading dye and appropriate control samples and test samples. The gel was run at 150mV 200mA for 30 minutes and images were viewed using Easy Image analyser.

2.4 Flow cytometry

Mouse spleen and mesenteric lymph nodes (MLNs) were harvested. Both spleen and lymph nodes were crushed using the frosted ends of glass slides. A homogenous suspension was prepared using FACS Buffer (Table 2.4). Cells were collected after centrifuging at 1500rpm for 5 minutes at 4°C in Allegra® X15R (Beckman Coulter, U.S.A.). The red blood cells (RBCs) were removed by resuspending the spleen samples in 10mL of RBC Lysis Buffer and incubating at 37°C for 10 minutes. RBC free samples were then washed in FACS Buffer. Around 2 x 10^6 cells were stained with 50µL of antibodies for 25 minutes on ice. Samples were then washed in 2mL of FACS Buffer as per the above centrifuge settings. Where purified antibodies were used, samples were blocked with 10% rat serum (Sigma-Aldrich®, Catalogue No: R9759) to prevent non-specific background interference.

For intracellular staining, the FOXP3 Fix/Perm Buffer kit was used as per the manufacturer's instructions (BioLegend®, Cat no: 421403). Secondary antibodies are listed in Table 2.3. Cells were stained with T cell and B cell markers (Table 2.3) to detect the presence of respective population. Prior to data acquisition, samples were filtered using 48µm nylon meshes (Sefar, 03-50/31). Flow cytometric data were acquired using CyAn™ ADP Flow Cytometer (Beckman Coulter, USA) and analysed with Flow Jo version 10 (Tree Star Inc., U.S.A.) for Windows.

Table 2.3: List of flow cytometry antibodies used

Antibody	Manufacturer	Dilution	Catalogue number
CCR6 PE (Clone 29-247)	BioLegend®	1:100	129-804
Goatα-Rat IgG Alexa Fluor® 488 (Secondary antibody)	JacksonImmunoResearch Laboratories,Inc.	1:200	A-11006
IL-17 Alexa Fluor 488 (Clone: TC11-18H10) Intracellular	BD Biosciences	1:100	17-5773-82
FOXP3 APC (Clone: FJK-16s) Intracellular	eBioscience	1:200	552094
B220 (CD45 R) APC Cy™ 7 (Clone: RA3-6B2)	BD Biosciences	1:200	HM3628
TCRαβ PB (Clone: H57-597)	Life Technologies™	1:200	H57-597
CD4 PerCP-Cy™5.5 (Clone RM4-5)	BD Biosciences	1:400	550954

Table 2.4: Buffers used in flow cytometry assays

FACS Buffer	100 mL 10x PBS
	1g BSA
	2 mL 10% Sodium Azide (Sigma-Aldrich®, S2002)
	H_2O to 1 L
RBC Lysis Buffer	4.55g NH_4Cl (Sigma-Aldrich®: A0171)
	10mL 1M HEPES (Sigma-Aldrich®, H3375)
	4.55g NH_4Cl (Sigma-Aldrich®: A0171)
	10mL 1M HEPES (Sigma-Aldrich®,H3375)
	H_2O to 500 mL

2.5 Real-time polymerase chain reaction (qPCR)

Colon tissues were homogenised on ice using disposable Omni Tip™ Plastic Generator Probes. RNA was extracted using Qiagen RNeasy mini kit (50) (Catalogue No: 74104), and an on-column DNase digestion was performed using RNase-Free DNase Set (Qiagen; Catalogue no: 79254) following the manufacturer's protocol and eluted using 20µl of RNA. The purity of eluted RNA was assessed using the ratio of absorbance at 260nm/280nm determined with an Eppendorf BioPhotometer (Eppendorf); samples above 1.8–2 were utilised for qPCR. A 2x TaqMan® Fast Advanced Master Mix (Catalogue No: 4444557), 2µl of the template was used in a reaction volume of 20µl (25ng per reaction). qPCR was performed on StepOnePlus^{TM}, StepOne Analysis software (version 2.2.3, Applied Biosystems). TaqMan primers (Table 2.5) were used in a reaction volume of 20µl (25ng per reaction). The PCR plate was run (Table 2.6) on Applied Biosystems StepOnePlus™ Real-Time PCR Systems (version 2.2.3). All samples were normalised to the control tissue sample and analysed using the $2^{-\Delta\Delta CT}$ method [162].

Table 2.5: List of TaqMan qPCR Primers

Primers	ID	Reference sequence
Il-10	Mm00439614_m1	NM_010548.2
Il-6	Mm00446190_m1	NM_031168.1
Il-22	Mm0044241_m1	NM_020525.4
Il-1β	Mm00434228_m1	NM_008361.3
Foxp3	Mm00475162_m1	NM_054039.2
Tgf-β1	Mm01178820_m1	NM_011577.1
Il-23a	Mm01160011_g1	NM_031252.2
Eef2	Mm01171434_g1	NM_007907.2
Ccr6	Mm99999114_s1	NM_009835.4
Ccl20	Mm01268754_m1	NM_016960.2

Table 2.6: Thermal cycling conditions for real-time PCR

Applied Biosystems Real-Time PCR System	**Thermal cycling profile**			
	Parameter	**Polymerase activation**	**PCR (40 cycles)**	
		Hold	**Denature**	**Anneal/Extend**
	Temp. (°C)	95	95	60
StepOnePlus™ system	**Time(mm:ss)**	00:20	00.01	00:20

2.6 Explant culture

Medium for culturing colonic explants was prepared using 500µL of complete RPMI 1640 (Gibco ™, In Vitro Technologies Australia Pty Ltd) supplemented with 10% v/v foetal calf serum (FCS; Gibco, Life Technologies, Australia Pty Ltd), penicillin (100 mU/L) and streptomycin (100 mg/L) (Sigma-Aldrich Pty Ltd, NSW, Australia) was dispensed in a 24-well culture plate and kept on ice. Once the tissues were harvested they were weighed and then cultured for 24 hours by incubating at 37℃ with 5% CO_2. After the incubation period the supernatants were collected from the culture plate and stored in -80℃ until further analysis.

2.7 T cell stimulation assay

2.7.1 Antibody coating

Antibody coating for the assay plates was prepared according to the manufacturer's protocol, a 5-10 µg/mL solution of anti-CD3e (Clone: 145-2C11, Catalogue No: 14-0031-82, Size 100µg) in sterile PBS, and the assay was performed in duplicates. A volume of 50µL of anti-CD3e antibody solution was dispensed to the experimental wells in the 96-well assay plate. For the control unstimulated wells, 50 µL of sterile phosphate buffered saline (PBS) was added and the plate covered tightly with Parafilm^{TM} to avoid sample evaporation and kept at 4°C overnight. Prior to the addition of cells, 50µL anti-CD3e antibody solutions were pipetted out of each well on the 96-well plate.

2.7.2 Enumeration of cells

MLNs were crushed by the frosted ends of glass slides, and the resulting cell suspension filtered using a 5ml round-bottom polystyrene test tube with cell strainer cap (12 x 75 mm, Catalogue No: FAL352235). Cell suspension was prepared and resuspended at a concentration of 1-2 x 10^6/mL in complete RPMI 1640 (Gibco^{TM}, Invitro Technologies Australia Pty Ltd) supplemented with 10% v/v foetal calf serum (Gibco, Life Technologies, Australia Pty Ltd), penicillin (100 mU/L) and streptomycin (100 mg/L) (Sigma-Aldrich Pty Ltd, NSW, Australia). The culture plate wells were rinsed with 200µl of phosphate buffer saline (PBS) before the addition of cells. Anti-mouse CD28 (Clone 37.51 grade, Catalogue No: 16-0281) was added to each well along with cell suspension in duplicates at a concentration of 2µg/mL (200µl/well), incubated at 37°C with 5% CO_2.

Cells were harvested after 24 hours and supernatants were collected after a spin at 200xg for 5 minutes. Supernatants were stored at -80℃ until used.

2.8 Cytokine assay

The explant supernatants and T cell supernatants were analysed using a custom-made Milliplex Mouse TH17 Magnetic Bead kit of analytes (IL-10, IL-17A, IFN-γ, IL-1β, CCL20 & IL-23). The samples were centrifuged (Heraeus Biofuge Stratos) at 4°C at 10,000xg for 15 minutes. The assay was performed according to the manufacturer's protocol on a Bio-plex 200 System (Bio-Rad Laboratories, Inc., Hercules, CA). A volume of 25µl of the undiluted sample was added to each well of the assay plate. The fluorescence intensity (FI) for each sample was acquired and analysed with Bioplex Manager 6.0. The explant colon tissue sample supernatants were assessed for cytokine levels. Cytokine levels in the supernatant were normalised to tissue weight to obtain pg/ml of cytokines/1 gm of tissue.

2.9 Histopathology

Colon tissues were Swiss-rolled, fixed overnight with 10% formalin and embedded in paraffin. Tissue sections were cut to an approximately 3–5µm thickness by a rotary microtome and were stained with Gill's haematoxylin and eosin (H&E). The H&E stained sections were imaged using an Olympus DP72 microscope. Dr Ruchira Fernando, the histopathologist involved in our study, modified the grading for colitis (Table 2.7) and scored the H&E tissue sections from all animals in a blinded manner. Cell counting was performed manually in 10 random microscopic fields at magnifications of 100x and 400x.

2.10 Data analysis

The data analysis was conducted by using Graph Pad Prism version 5.01 (Graph Pad Software Inc., U.S.A.). All data were expressed as a mean ± standard error of the mean (SEM). One-way ANOVA (non-parametric) was used and the comparisons between groups were analysed using Dunn's multiple comparison tests or Tukey's multiple comparison tests. A resulting *p*-value of $\leq$ 0.05 was considered significant.

Table 2.7 Scoring sheet for H&E sections

	PC	MC	DC
1.Distribution of inflammation			
0=none			
1= focal			
2=multifocal			
2.Depth of Inflammation			
1=Mucosal			
2=Submucosal			
3=Transmural			
3.Lamina propria neutrophils			
0= none			
1= 1-10			
2= 11-20			
3= > 20			
4.Cryptitis			
0= no cryptitis			
1= cryptitis			
5.Crypt abscess			
0= none			
1=1-5			
2=6-10			
3=>10			
6.Goblet cell loss			
0= normal <10% loss			
1= 10-25%			
2= 25-50%			
3= >50%			
Crypt architecture			
0= normal			
1= mildly irregular			
2= moderate			
3= severe			
Crypt shortening			
0=No damage			
1=25% shortening (150-200µm)			
2=50% shortening(200-250 µm)			
3=75% shortening(250-300 µm)			
4=100% shortening (>300 µm)			
Ulceration			
0= None			
1= Superficial			
2=Deep			

Chapter 3

Generation of Winnie x *Ccr6*$^{-/-}$ mice

3.1. Introduction

Inflammatory bowel disease (IBD), a chronic intestinal inflammation, is reportedly developed by modulations in the intestinal immune responses which are determined by a complex interaction between environmental, genetic and microbial factors [163]. Animal models are used to mimic IBD in order to better understand the disease mechanisms and attempt to describe its aetiology. Recent advances in this field of research have been brought about by the use of animal models in which inflammation is developed by chemical induction or by genetic alterations [121,164]. Chemicals such as DSS, TNBS, and oxazolone are used to induce intestinal inflammation initiated by mucosal damage leading to either acute inflammation or chronic inflammation. There are a number of disadvantages associated with the use of chemicals, such as optimising dosages and differences in microflora, all leading to substantial variability in these factors [121].

Genetic alterations are the alternative approach to chemically induced models of IBD as they assist in the development of chronic inflammation. The provision for genetic diversity and manipulations within the mouse genome facilitates applications critical for defining a gene function, gene mutation and/or gene inactivation *in vivo* [165,166]. Gene targeting was implemented to develop knockout mouse models, wherein thousands of genes have been knocked out in mice [165,167-169] Using modern techniques for gene

targeting or trapping, a knockout model for each mouse gene is ongoing globally [169]. Inactive genes or gene mutations are many a time responsible for human diseases. Genetic mutations are often demonstrated or reproduced using a mouse for studying diseases.

Apart from being a good physiological analogue to humans, the size, lifespan and prolific nature of mice render them a valuable research tool [165]. Knockout mice proved to be a useful resource when studying a gene's role in a specific gene deletion model. The phenotype of a knockout mouse gives an insight into the functions of the gene *in vivo*. Knockout models are imperative in identifying strategies to develop therapies for treating diseases [170]. Around 60 mouse models have been developed to date and are used to study IBD pathogenesis [119].

One such mouse model, Winnie, known to closely mimic human ulcerative colitis, was produced using random ENU mutagenesis reported by Heazlewood et al. (2008). Mucin-2 is a major component of the intestinal barrier, produced by goblet cells. Mice develop spontaneous colitis by a single missense mutation in the Muc2 gene of the mucin protein leading to the misfolding of mucin, causing ER stress. The homozygous Win/Win phenotype displays a spontaneous colitis phenotype similar to ulcerative colitis in IBD patients with a Th17-dominated immune response [43].

Genome-wide association studies reported that the CCR6 gene is one among the 30 genomic loci implicated in IBD. CCR6 is a β-chemokine–specific receptor for CCL20 (MIP-3α/LARC/Exodus) in both humans and mice. The CCR6-CCL20 chemokine axis reportedly plays a major role in many diseases such as rheumatoid arthritis, EAE, and many other immune-mediated diseases, including IBD. CCR6 is the sole receptor for CCL20 and the function of this axis during inflammation remains unclear. Using gene

targeting techniques, *Ccr6*$^{-/-}$ mice were generated in order to study this issue [137,171]. *Ccr6*$^{-/-}$ mice displayed reduced numbers of M cells and smaller Peyer's patches, resulting in a reduction in the number of domes [49,172]. CCR6 is involved in the differentiation of B cells and Th17 lymphocytes [32]. Both Th17 and Treg cells express CCR6. Research studies suggest that the dynamic balance elicited by the CCR6-CCL20 axis between pro-inflammatory Th17 cells and anti-inflammatory Treg cells either determines the development of inflammation or maintains a homeostatic environment. Studies using *Ccr6*$^{-/-}$ mice suggest that the absence of the *Ccr6* gene affects the Treg subset population more than the Th17 subset population [32]. Hence the focus of this study was to utilise and *Ccr6*$^{-/-}$ mice to develop a mouse model in order to study colitis and the changes associated with the absence of *Ccr6*.

3.2. Aims /hypothesis

The initial aim of this study was to generate and identify Winnie x *Ccr6*$^{-/-}$ mouse model by crossbreeding and backcrossing Winnie and *Ccr6*$^{-/-}$ mice. The hypothesis was that the Winnie and *Ccr6*$^{-/-}$ mice when crossed would inherit the mutations from both genotypes.

3.3. Results

3.3.1 Generation of Winnie x *Ccr6*$^{-/-}$ mice

Based on the Mendelian pattern of inheritance, the strains of Winnie and *Ccr6*$^{-/-}$ were set up to breed mice for this study. Winnie mice were imported to our breeding facility where they were rederived to produce specified pathogen-free (SPF) mice for breeding purposes. In our animal facility, the rederivation process is a standard procedure used to obtain SPF mice. A breeding plan was sketched to generate Winnie x *Ccr6*$^{-/-}$ mice, and the heterozygous strains of Winnie (Win/WT) mice were rederived by crossing with WT

strain (Figure 3.1). This initial step was expected to produce 50% Winnie and 50% WT. Subsequently, heterozygous Winnie (Win/WT) mice were crossed with heterozygous Winnie mice and were expected to produce 25% homozygous, 50% heterozygous and 25% wild type. In our third step, Winnie homozygous mice were crossed with *Ccr6* homozygous mice, and all pups produced were Winnie heterozygous and *Ccr6* heterozygous. Subsequently, in our fourth step, heterozygous Winnie mice and heterozygous *Ccr6* mice were backcrossed with Winnie heterozygous and heterozygous *Ccr6* mice, resulting in the generation of the following 9 different genotypes (Table 3.1). Winnie homozygous x *Ccr6* homozygous mice were generated.

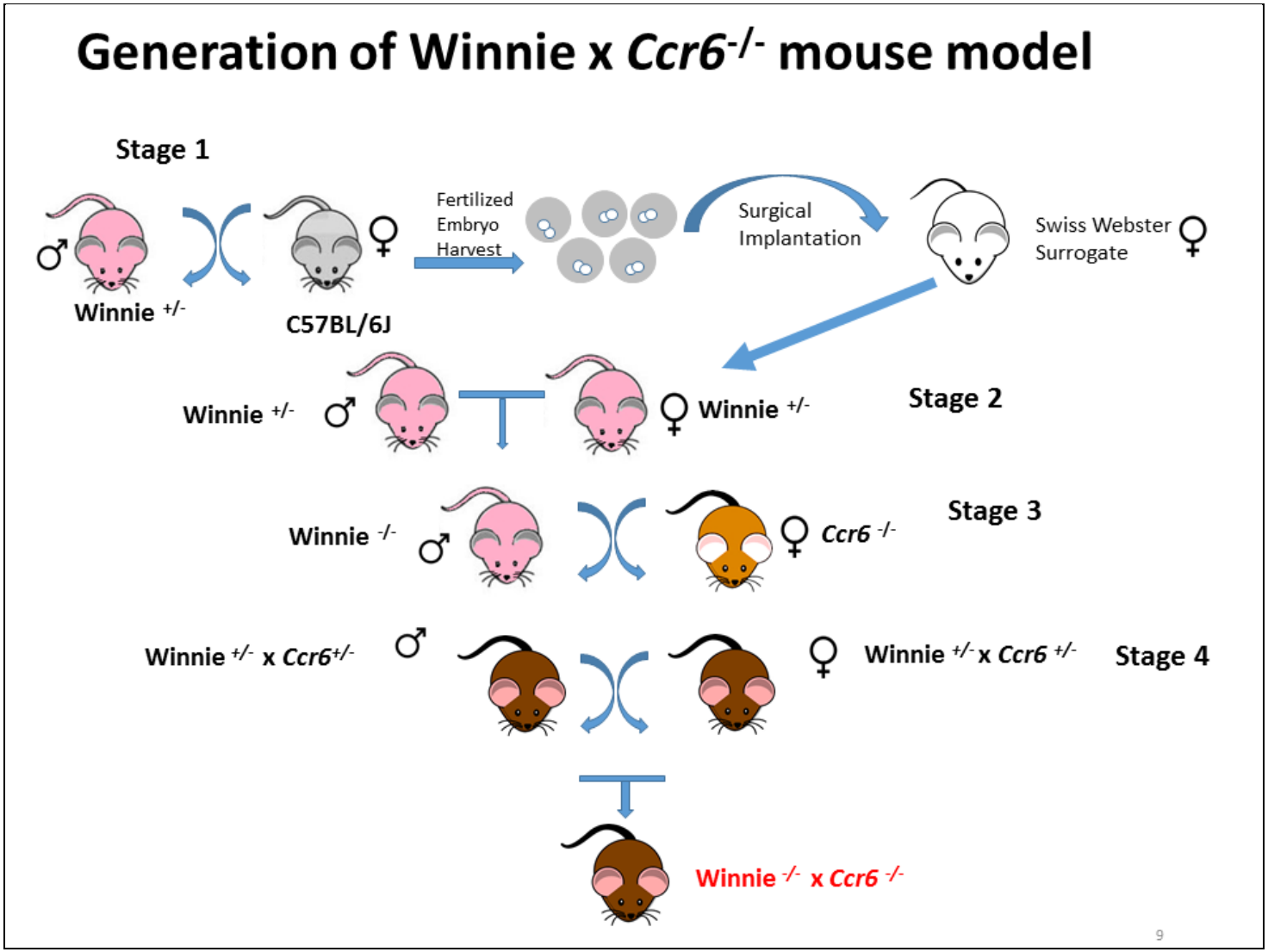


Figure 3.1: Generation plan for Winnie x *Ccr6*$^{-/-}$ mice.

A generation plan was outlined, based on the Mendelian pattern of inheritance for rederivation of Winnie mice, followed by crossbreeding and a backcrossing plan for the Winnie x *Ccr6*$^{-/-}$ mice is shown in the above flowchart.

Table 3.1 Percentages of genotypes obtained in the Winnie x *Ccr6*$^{-/-}$ generation.

Stages	Genotype				
	Heterozygous (Het)	Homozygous (Hom)	Wild-type (WT)	Mixed	Ratio
1		50% Winnie	50% WT		1:1
2	50% Winnie	25% Winnie	25% WT		1:2:1
3	50% Winnie Het; 50% *Ccr6* Het				1:1
4	25% Winnie Het; *Ccr6* Het	6.25% Winnie Hom; *Ccr6* Hom	6.25% Winnie WT; *Ccr6* WT	12.5% Winnie WT; *Ccr6* Het	1:9
				12.5% Winnie Het; *Ccr6* Hom	
				12.5% Winnie Hom; *Ccr6* Het	
				12.5% Winnie Het; *Ccr6* WT	
				6.25% Winnie Hom; *Ccr6* WT	

3.3.2 Winnie x *Ccr6*$^{-/-}$ genotyping

Genotyping was performed for each batch of pups to determine the respective genotype. Appropriate genotyping assays were then performed and homozygous Winnie and homozygous *Ccr6* mice were identified. The homozygous (Win/Win) Winnie mice were identified using TaqMan SNP assay. A custom-made SNP for Winnie mutant strain detection using TaqMan probes was designed for the Winnie genotyping assay by Life technologies where the reporter/quencher for Allele-1 was VIC/MGB-FAM, Allele 2-FAM/MGB-NFQ (Figures 3.2 & 3.3). In the process of identifying the mutant alleles for Winnie, the automated analysis in Step One Plus software was utilised. Optimisations of Winnie and *Ccr6*$^{-/-}$ mice were performed until a working protocol was developed for our study. For *Ccr6* genotyping, PCR was performed using the primers outlined in the 'Materials and Methods' chapter of this thesis (Table 2.5). The products were run on an agarose gel to identify the homozygous *Ccr6* genotype. A DNA easy ladder was used to assist in identifying the fragments, and the size of fragments was 250 base pairs (bp) for homozygous *Ccr6* and 350 base pairs for WT (Figure 3.4).

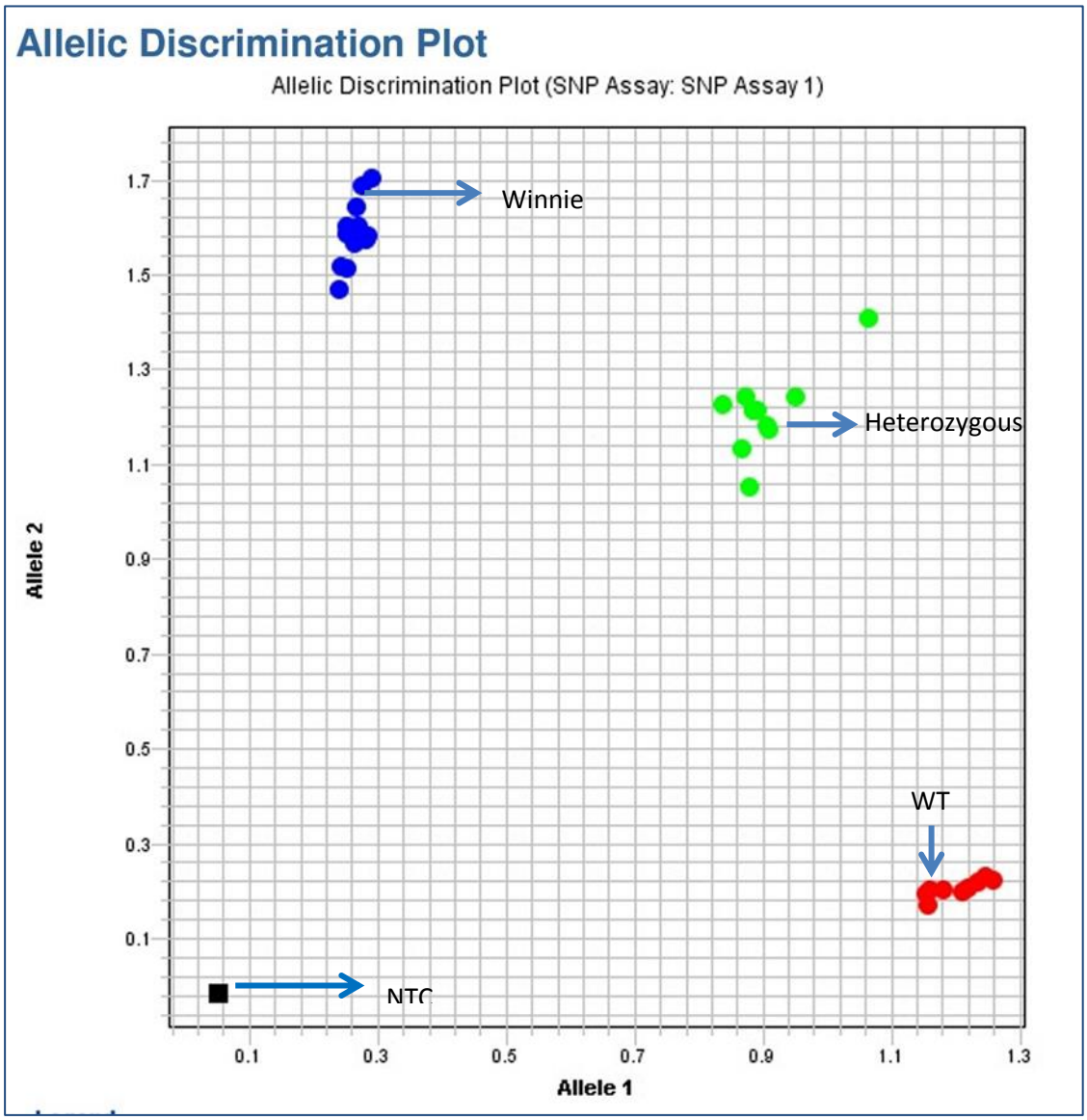


Figure 3.2 Distribution of allelic discrimination plots of genotypes.
Allelic discrimination plot of SNP assay shows clusters of blue dots which represent Allele 2/Allele 2 (Win/Win) homozygous Winnie mutants. Allele 1/Allele 2 (Win/WT) heterozygous is represented as a green cluster of dots, and Allele 1/ Allele 1 wild-type (WT) is represented as red dots. No template control (NTC) is represented in black. The SNP assay was performed using neat samples and appropriate controls for each genotype.

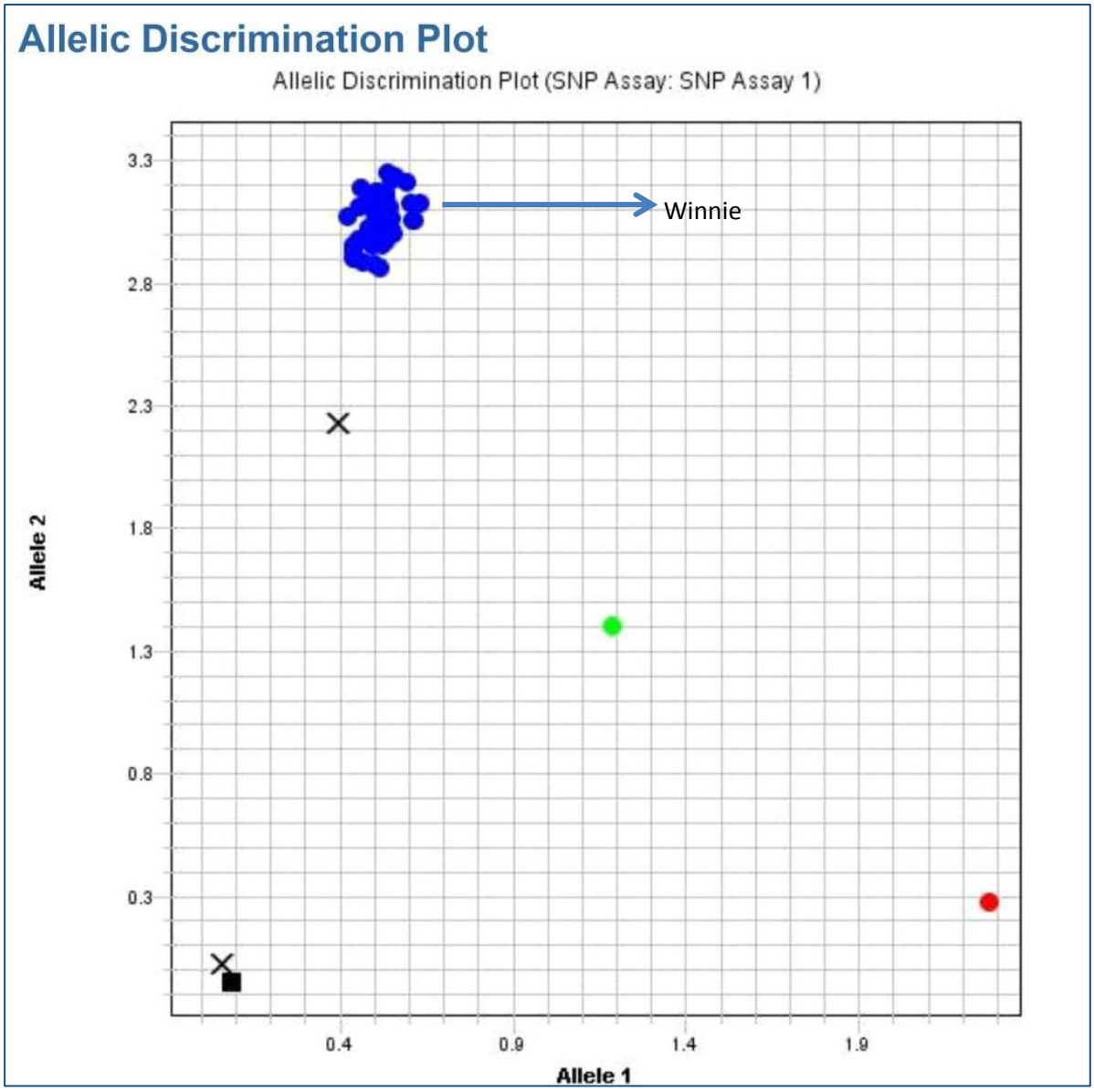


Figure 3.3 Homozygous Winnie allelic discrimination plots
Allelic discrimination plot showing clusters of homozygous Winnie mice represented by blue dots. Heterozygous control represented by a green dot, WT control represented by a red dot and a no template control is shown in black. A complete population of homozygous Winnie (Win/Win) was identified.

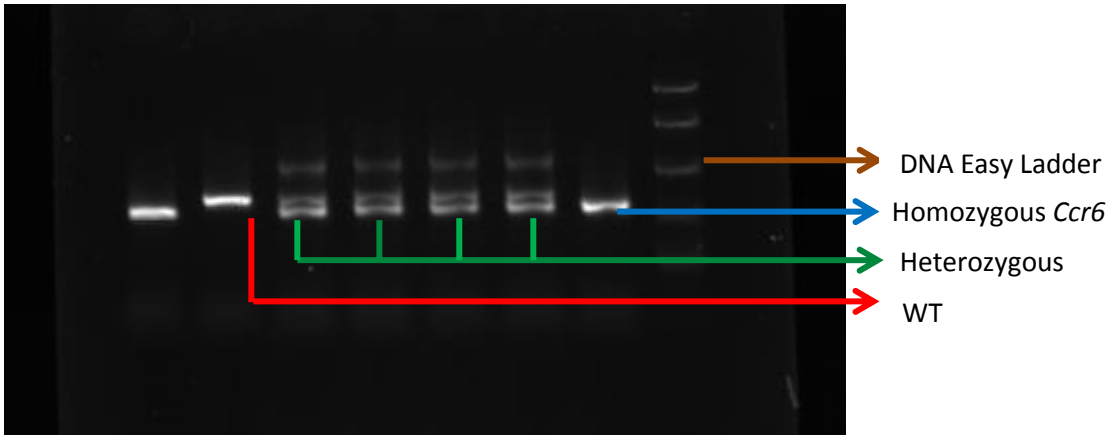


Figure 3.4: *Ccr6*$^{-/-}$ genotyping amplicons.
Agarose gel electrophoresis was performed and the amplified fragments were visualised on 2% agarose gel using SYBR safe DNA gel stain. A DNA easy ladder was used, product size band-100-2000 bp. The product size was 250 bp for homozygous *Ccr6* and 350 bp for WT.

3.4 Discussion

Rederivation of the Winnie mouse strain was performed to achieve SPF mice for our breeding program. Rederivation is a standard laboratory practice performed to maintain the health status of animals in our animal facility. Imported mice are always subjected to the rederivation procedure when procured from vendors or from other research labs which differ in veterinary health surveillance systems. The rederivation process assists in achieving 'cleaned' or decontaminated laboratory animals, which do not contain certain pathogens or any transmissible zoonotic diseases, thereby preventing the disease from spreading into the new housing facility [173]. Embryos were harvested for the process of rederivation and surgically implanted into the surrogate mice. After the surrogate mice were screened negative for unacceptable pathogens, the pups were considered as pathogen-free and were subsequently used for breeding purposes [173].

A mutant mouse strain, Winnie, was chosen for this study as this particular mouse strain displays non-complementing missense mutation in the *Muc2* mucin gene. The mutation resulted in the development of mild distal intestinal inflammation in the Winnie strain.

Colitis was seen to develop in Winnie mice as early as within 6 weeks. Winnie colitis reportedly displays a disease similar to the ulcerative colitis-like phenotype. Increased intestinal permeability in Winnie mice further resembles disease as in ulcerative colitis in humans [43]. The single nucleotide polymorphism (SNP) is an alteration from a cysteine to tyrosine in the N-terminal D3 domain of *Muc2* of Winnie mice. The aberrant oligomerization of *Muc2* resulted in ER stress and subsequently initiated UPR, eventually leading to spontaneously occurring colitis. Unlike other contrived models, Winnie mice develop spontaneous chronic colitis due to defective *Muc2* biosynthesis, followed by inflammation and goblet cell dysfunction, and hence were chosen to be used in developing the Winnie x *Ccr6*$^{-/-}$ model for this study. *Ccr6*$^{-/-}$ mice reportedly have an altered intestinal immune system, underdeveloped Peyer's patches and absence of isolated lymphoid follicles (ILF) [137,174]. In the intestinal mucosa, the *Ccr6* gene was reportedly a major regulator of lymphocyte homeostasis and humoral immunity [137,140]. Varona et al. (2001) suggested that since CCR6 is involved in both homeostasis and inflammation, *Ccr6*$^{-/-}$ mouse models are a useful tool for studying inflammatory diseases such as IBD and hence this model was chosen for this study.

A breeding program (Figure 3.1) was created for the generation of Winnie x *Ccr6*$^{-/-}$ mice, the rederivation process of Winnie mice with WT mice was performed and the embryos were aseptically transferred and surgically implanted into surrogate Swiss Webster mice. Winnie heterozygous (Win/WT) mice were then mated with Winnie heterozygous (Win/WT) mice which subsequently produced 50% homozygous and 50% heterozygous pups. In the next stage, homozygous Winnie mice were then crossed with *Ccr6* heterozygous mice which resulted in heterozygous pups for both Winnie and *Ccr6*.

Backcrossing of heterozygous Winnie mice and heterozygous *Ccr6* with heterozygous Winnie and *Ccr6* mice resulted in pups of 9 different genotypes (Table 3.1). The homozygous Winnie mice and *Ccr6* homozygous mice were subsequently used to generate our Winnie homozygous x *Ccr6* homozygous experimental mice.

Genotyping was performed to identify the respective genotypes of the pups produced in each litter. An SNP assay for genotyping was performed as described previously, and the SNP assay detected the SNP in Winnie mice and identified the alleles for all respective genotypes and the Winnie mutant. The genotype of each mouse was identified by allelic discrimination plots (Figures 3.2 and 3.3) using an SNP assay for Winnie mice, and *Ccr6* $^{-/-}$ genotyping was performed by PCR and the resulting amplicons were identified by agarose gel electrophoresis (Figure 3.4) to confirm the *Ccr6* gene deletion.

Optimisation of the genotyping protocol for both Winnie and *Ccr6*$^{-/-}$ was performed and appropriate protocols were subsequently utilised for identification of genotypes. Results of genotyping were analysed by using appropriate Step One software for Winnie mice and confirmed accordingly. The amplicons of *Ccr6*$^{-/-}$ mice genotyping were run on an agarose gel, and a DNA ladder was used to identify the amplicons of WT/heterozygous/ homozygous *Ccr6* mice, eventually confirming their genotype according to the size of the amplicons. A ratio of 1:9 as per Mendelian inheritance pattern was observed in obtaining the required Winnie x *Ccr6*$^{-/-}$ genotype. Many litters were produced until a working population of experimental mice resulted, and these were subsequently utilised for experimental purposes whilst maintaining healthy breeding pairs.

3.5 Concluding remarks

In this study, generation of a Winnie x *Ccr6*$^{-/-}$ mouse model and identification of homozygous genotype of the same was achieved. An appropriate genotyping technique for each mouse strain was optimised and utilised. The respective genotypes were confirmed by performing an SNP assay for Winnie mice and PCR followed by agarose gel electrophoresis for *Ccr6*$^{-/-}$mice. Generation of Winnie x *Ccr6*$^{-/-}$ mice was achieved; further assessments of its phenotype and its effects on inflammation in the absence of *Ccr6* would be a valuable asset in understanding the role of CCR6 in the disease process of IBD.

Chapter 4

Clinical assessment and histopathological examination of Winnie x *Ccr6*$^{-/-}$ mice

4.1 Introduction

An important focus of our study was the assessment of the novel model Winnie x *Ccr6*$^{-/-}$ phenotype. In this chapter, phenotype assessments of Winnie x *Ccr6*$^{-/-}$ mice pertaining to clinical parameters and changes in the histopathology of this strain are outlined. The Winnie mice phenotype has been extensively studied based on clinical, histological, molecular and immunological aspects, and it is a well-established spontaneous chronic colitis model [43,145,175]. It is imperative to validate the phenotype of any experimental mouse strain as it ascertains the utility of that animal in the field of biomedical research.

A systematic approach for the assessment of phenotype of experimental mice which may have been genetically altered was outlined by Wood et al. [176]. Primary-level and secondary-level assessments were suggested as a guideline to assist in the phenotype assessment process of experimental animals, precisely mice or rats. The primary-level assessments are basically to identify abnormalities, whilst the secondary-level assessments are for quantification and evaluation of abnormalities. Both primary and secondary phenotype assessments are vital in studying the current mouse models used for investigating diseases in biomedical research [176].

The phenotype and colitis assessments in Winnie x *Ccr6*$^{-/-}$ mice were performed based on previously established protocols [131]. The specific parameters included the evaluation of colon weight, colon length, and colon weight by body weight ratio. Colon weight is regarded as a significant indicator of colitis, and the histopathology of colon tissue sections and small intestine stained with H&E were examined and graded using a blinded grading system developed for intestinal inflammation based on the standard protocol of histological scoring for colitis [131,177]. In the present study, the phenotype evaluation process noted other general aspects with regard to the experimental animal's general well-being as part of the assessment process.

Ccr6$^{-/-}$ mice were developed to study various diseases such as RA, psoriasis and EAE [91,109]. *Ccr6*$^{-/-}$ mouse models are also considered a very important tool in understanding lung and gut diseases. *Ccr6* is implicated as a susceptibility locus in CD and the role of CCR6-mediated colitis has been advanced by the development of *Ccr6*$^{-/-}$ mouse models in order to study the disease process of IBD *in vivo*. Due to the absence of *Ccr6*, the development of disease and the alteration of immune responses can be studied by using *Ccr6*$^{-/-}$ mice. Defective leukocyte homeostasis and changes in the cytokine milieu in the intestinal mucosa are attributed to the absence of *Ccr6* [98]. Winnie, a spontaneous chronic colitis mouse model develops mild distal colitis by 6 weeks of age [145].

Colonic inflammation displayed by the Winnie strain is due to the homozygous missense point mutation in the mucin 2 gene (*Muc2*). The resulting endoplasmic reticulum stress leads to the development of colonic inflammation similar to that of ulcerative colitis seen in humans [43]. The SNP is the nominal Winnie mutation, and the substitution of cysteine with tyrosine in the D3 domain at the N terminus of Muc2 was identified in Winnie mice [43]. The goblet cell dysfunction occurs due to the mutation in Winnie mice and, unlike

other contrived models, colitis spontaneously occurs in this phenotype as a result. There is a significant knowledge gap that exists regarding our understanding of the exact disease processes contributing to both UC and CD in IBD. The Winnie mouse model, which exhibits colonic inflammation due to a primary epithelial cell defect, ascertains the need for the secretion of mucin in order to maintain the integrity of the secretory cell, thereby preventing the occurrence of colonic inflammation.

The crosstalk between the colonic epithelial cells and luminal microbes, followed by the mucosal immune responses that occur during the colonic inflammation, needs to be addressed in order to understand the disease pathogenesis and progression. The reciprocal paracrine interaction of epithelial cells and immune cells in the intestine needs to be understood in order to relate or mimic IBD *in vivo*. The involvement of both the adaptive and innate immune system is well showcased in Winnie mice colitis. Hence serves as a potential mouse model for spontaneously occurring colitis to explore the role of the *Ccr6* gene in this colitic context.

4.2 Aims/hypothesis

Winnie mice develop mild spontaneous distal colitis due to a primary epithelial defect, leading to impairment in goblet cell function. The present study aimed to generate an experimental genotype, thereby intended to document physiological changes pertaining to colitis and to investigate the initiation and development of inflammation during the absence of the *Ccr6* gene both clinically and histologically. The hypothesis of this study was that *Ccr6*$^{-/-}$ mice, when crossed with Winnie mice, results in exacerbation of colitis due to the absence of the *Ccr6* gene.

4.3 Results

4.3.1 Assessment of clinical parameters

As part of the clinical assessments, all mice aged 8–12 weeks and of either sex were utilised for this study. The body weight of mice (n=9) from all four genotypes (Winnie 16–20g, Winnie x *Ccr6*$^{-/-}$ 18–25g, *Ccr6*$^{-/-}$ 22–28g and WT 19–20g) were noted prior to sacrifice. Winnie x *Ccr6*$^{-/-}$ mice, the experimental mouse model used in this study, were compared with the other three groups of mice. The parameters for evaluation of intestinal inflammation were based on previously established protocols [131] (Table 4.1).

The body weight of Winnie x *Ccr6*$^{-/-}$ mice was compared with WT, Winnie, *Ccr6*$^{-/-}$ mice (Figure 4.1). It was noted that the body weight of Winnie x *Ccr6*$^{-/-}$ mice were decreased (P-value <0.05) relative to *Ccr6*$^{-/-}$ mice, and the body weight of Winnie mice was decreased (P-value <0.001) relative to *Ccr6*$^{-/-}$ mice. The body weight reduction observed in Winnie x *Ccr6*$^{-/-}$ mice indicates that this could possibly be due to the genetic ablation of *Ccr6* in Winnie mice.

Table 4.1: Protocol for evaluation of intestinal inflammation

Parameters	Procedure/Details
Body weight	Mice weighed prior to culling
Colon length	Measured from the caeco-colic junction up to rectum
Colon weight	Weight of wet colon
Histological colitis scores	Blinded grading of H&E stained specimens

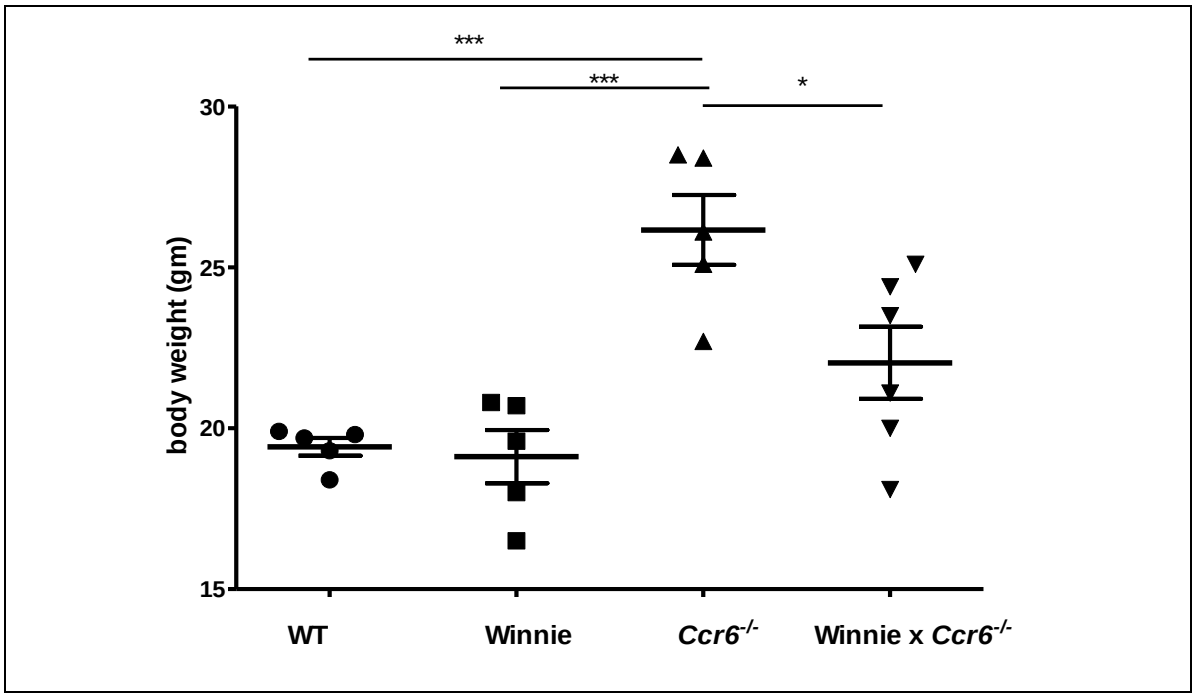


Figure 4.1: Genetic ablation of *Ccr6* results in alteration of body weight in Winnie x *Ccr6*$^{-/-}$ mice.
Body weight of WT, Winnie, *Ccr6*$^{-/-}$ and Winnie x *Ccr6*$^{-/-}$ mice aged 8–12weeks was measured prior to culling. The body weight between all four genotypes was compared using one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. A *significance is shown by $p < 0.05$, data represents mean± SEM (n=9 / genotype).

4.3.2 Colon length

The large intestine was retrieved from the body cavity of mice and was excised near the caeco-colic junction. The colon from Winnie, *Ccr6*$^{-/-}$, and Winnie x *Ccr6*$^{-/-}$ was retrieved the same way and measured for length from the caeco-colic junction up to the proximal rectum. The colon length between the four genotypes did not vary (Figure 4.2A), and measurements were the following: WT–7.3 cm, Winnie –7.7 cm, *Ccr6*$^{-/-}$ – 7.6 cm and Winnie x *Ccr6*$^{-/-}$ –7.1 cm. The colon length was compared and results did not show any difference (Figure 4.2B) between the four groups and failed to achieve a statistical significance (n=9 / genotype).

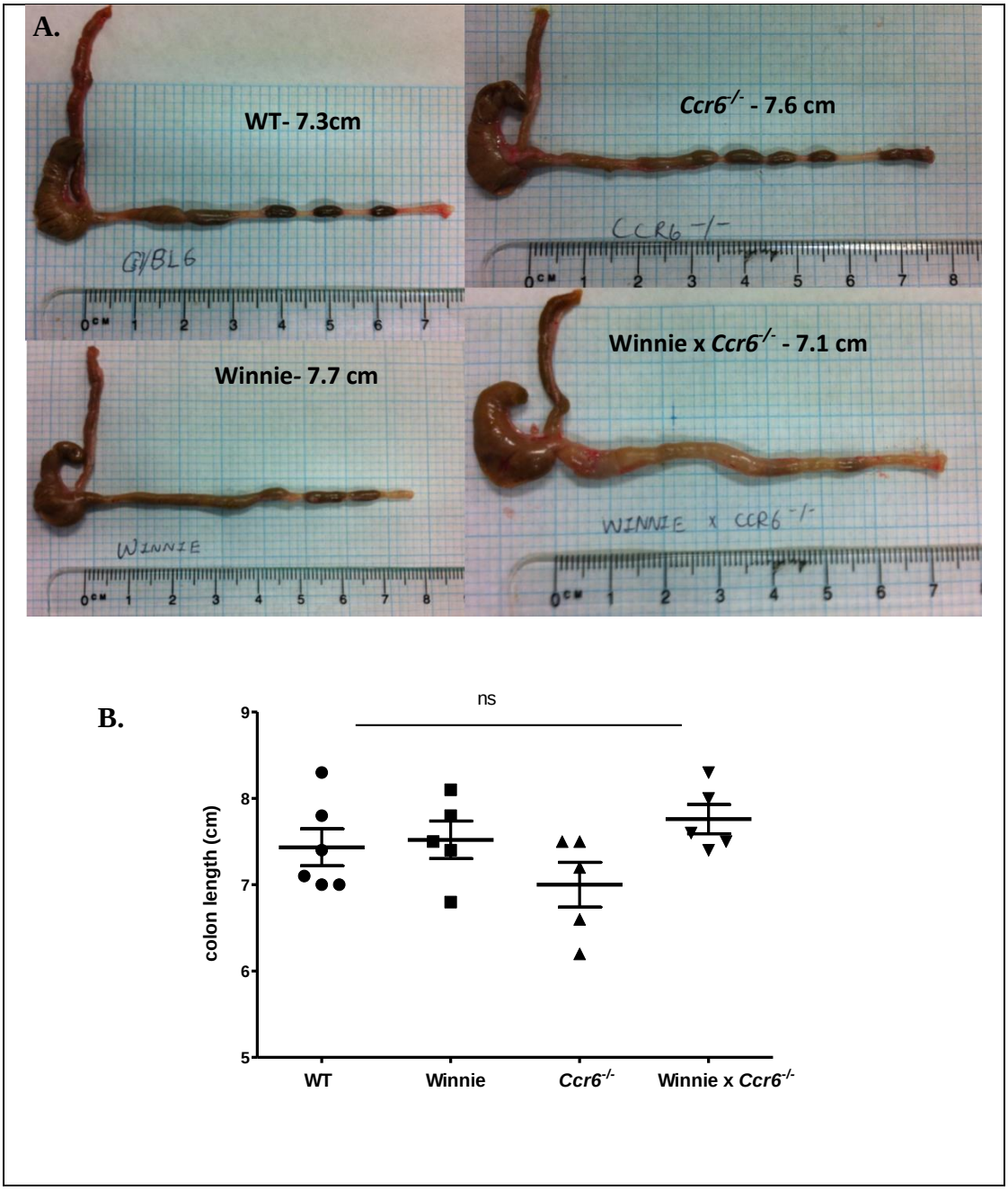


Figure 4.2: Absence of *Ccr6* does not alter colon length in Winnie x *Ccr6*$^{-/-}$.
A. Colon length was measured from (WT - 7.3 cm, Winnie - 7.7cm, *Ccr6*$^{-/-}$ - 7.6 cm, Winnie x *Ccr6*$^{-/-}$ - 7.1cm) mice aged between 8–12 weeks.
B. Colon length between four genotypes was compared using one-way ANOVA and Tukey's multiple comparison tests. Results showed no difference between the four genotypes, (ns) no statistical significance was shown, data represents mean± SEM (n=9 / genotype).

4.3.3 Colon weight

The wet colon was cut open longitudinally and the faecal contents were removed and weighed. The colon weight of each mouse from all four genotypes, WT, Winnie, *Ccr6*$^{-/-}$ and Winnie x *Ccr6*$^{-/-}$ mice was noted. The colon weight (Figure 4.3A) of Winnie x *Ccr6*$^{-/-}$ was significantly increased (P-value < 0.01) relative to *Ccr6*$^{-/-}$ mice. However, the colon weight of WT (P-value <0.001) was lower than the *Ccr6*$^{-/-}$ mice. The results indicated that, in the absence of *Ccr6*, colon weight was not significantly increased in Winnie x *Ccr6*$^{-/-}$ mice when compared to Winnie mice. The colon weight was analysed for the ratio of body weight (Figure 4.3B). The Winnie x *Ccr6*$^{-/-}$ mice colon exhibited an increase (P-value < 0.05) relative to *Ccr6*$^{-/-}$ mice. Winnie mice had an increase in the colon weight over body weight ratio (P-value <0.05) relative to *Ccr6*$^{-/-}$mice. The results suggested that in the Winnie x *Ccr6*$^{-/-}$ phenotype, there was no significant change in the ratio of colon weight over body weight due to the absence of *Ccr6*.

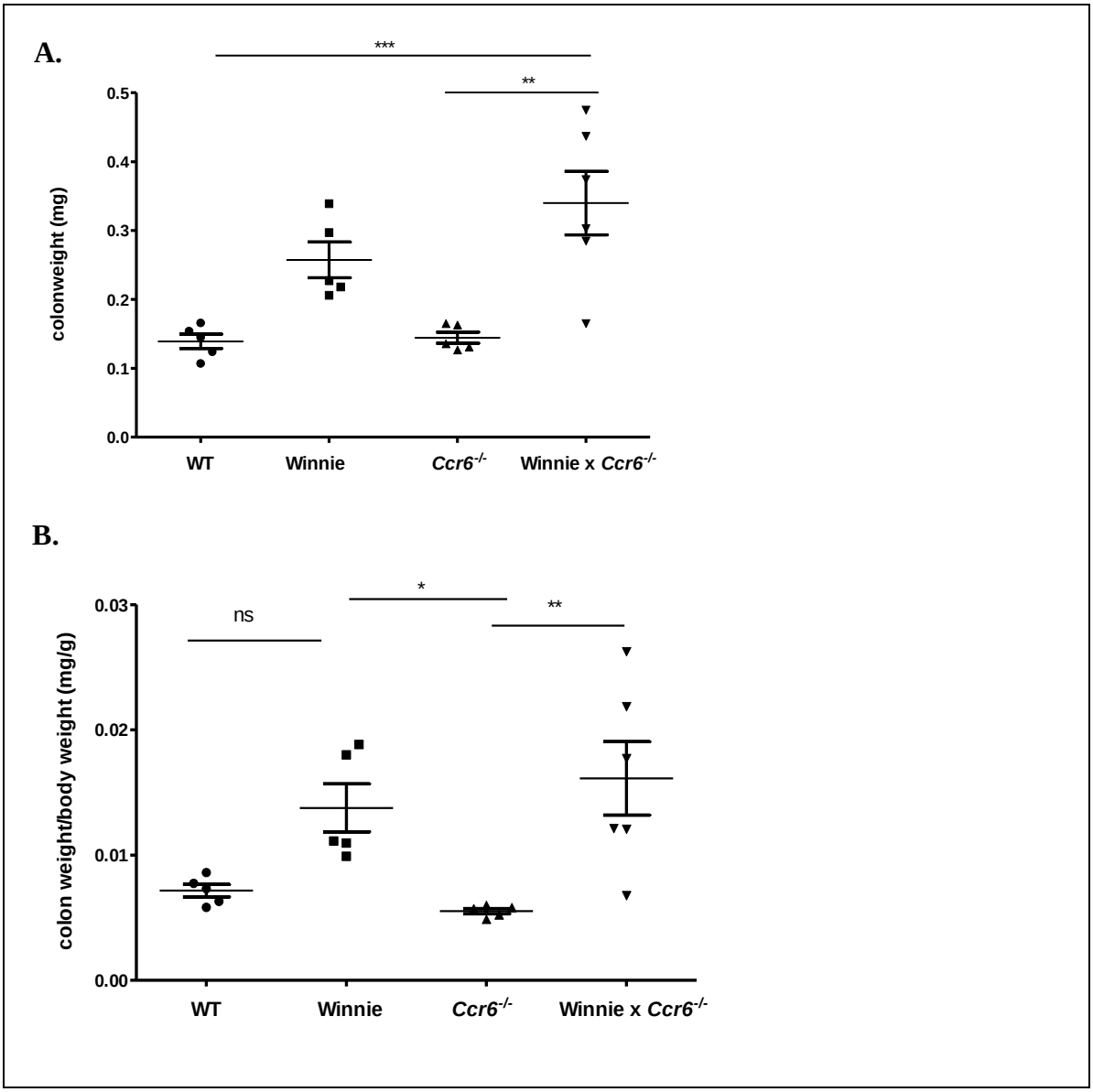


Figure 4.3: Increased colon weight and colon weight/body weight ratio in Winnie x *Ccr6*$^{-/-}$ relative to *Ccr6*$^{-/-}$ mice.

A. The wet colon was cut opened longitudinally; faecal contents were removed and weighed. The colon weight of each mouse from four genotypes, WT, Winnie, *Ccr6*$^{-/-}$ and Winnie x *Ccr6*$^{-/-}$ mice was noted and compared between them. One-way ANOVA and Tukey's Multiple Comparison test was used, a significance of $^{**}p < 0.01$, $^{***}p < 0.001$ was shown, data represents mean± SEM (n=9 / genotype).

B. The colon weights were presented as a ratio of body weight. Means were compared using one-way ANOVA and Tukey's Multiple Comparison tests, a significance of $^{*}p < 0.05$, $^{**}p < 0.01$ was shown, data represents mean± SEM (n=9 / genotype).

4.3.4 Histopathological assessment

Histopathological assessments were performed in a blinded fashion for H&E stained colon tissue sections using the grading system (Table 2.7) adapted from Geboes et al. [177]. The colon tissue section slides were examined and scored for features of inflammation, mucosal lymphoid aggregates, cryptitis and crypt elongation, goblet cell loss and crypt architectural changes. The examination of H&E stained tissue sections revealed (Figure 4.4) Winnie x *Ccr6*$^{-/-}$colon was highly inflamed in the proximal colon (PC), (P-value < 0.01) to mid colon (MC) relative to Winnie PC.

Though the distal colon (DC) in Winnie x *Ccr6*$^{-/-}$ seemed to appear mildly inflamed (P-value < 0.001) relative to Winnie mice distal colon. While DC was highly inflamed in Winnie mice, the proximal colon (P-value < 0.01) remained mildly inflamed. The *Ccr6*$^{-/-}$ mice colon and the WT mice colon, when examined, did not indicate any inflammation and appeared to be normal. The mid colon of Winnie x *Ccr6*$^{-/-}$ was highly inflamed (P-value < 0.05) relative to the Winnie mice mid colon. The Winnie x *Ccr6*$^{-/-}$ mice mid colon was inflamed (P-value < 0.05) when compared to its proximal colon.

Surprisingly, the deletion of *Ccr6* had not only exacerbated the colitis but had conferred differential distribution of inflammation in Winnie x *Ccr6*$^{-/-}$. Examinations of colon tissue sections revealed goblet cell depletion and reduction in mucus secretion concurrent with the Winnie phenotype (Figure 4.6). Colon sections also exhibited inflamed submucosa in the proximal colonic segment. Damage to the epithelium and changes in crypt architecture such as crypt shortening or crypt distortion were observed in the PC to MC regions of Winnie x *Ccr6*$^{-/-}$ mice (Figure 4.5) Neutrophil infiltrations were observed in the glands and an abundance of neutrophils in the tissues indicated active inflammation. An abundant presence of neutrophils in the proximal region was detected and reduced

numbers of neutrophils in the distal region, as opposed to that, observed in the Winnie mice phenotype (Figure 4.8). The occurrence of gland necrosis involving the PC and DC of Winnie x *Ccr6*$^{-/-}$ mice was also detected. The absence of inflammatory cell infiltration was observed in the submucosa of the DC.

Cryptitis was noted in the proximal colon of Winnie x *Ccr6*$^{-/-}$ and increased inflammation was also observed as the presence of crypt abscesses was detected in the mid colon segment. Neutrophil infiltration in the proximal colon extending towards the submucosal layer in Winnie x *Ccr6*$^{-/-}$ (Figure 4.7) mice was prominent. Hyperchromatic nuclei revealed epithelial damage and were observed in Winnie and Winnie x *Ccr6*$^{-/-}$ mice. More than 50% of goblet cell loss was detected in the Winnie DC along with some discrete crypt lesions and abscesses (Figure 4.8). Irregular crypt architecture was observed in the Winnie DC (Figure 4.6) and Winnie x *Ccr6*$^{-/-}$ PC (Figure 4.5). These results indicated an increase in inflammation in proximal to mid colonic segments in the Winnie x *Ccr6*$^{-/-}$ mice phenotype.

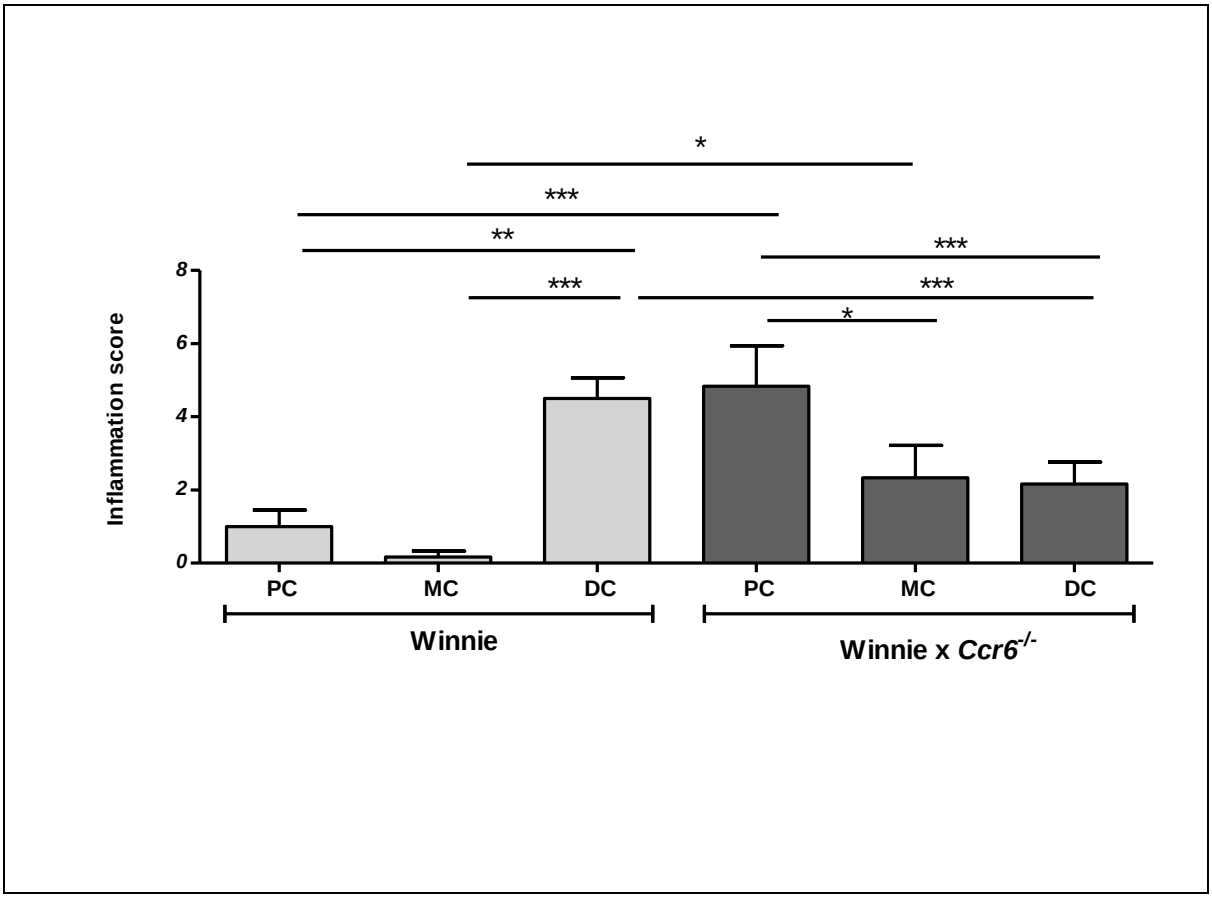


Figure 4.4: Increased inflammation in proximal to mid colon of Winnie x *Ccr6*$^{-/-}$.
H&E stained colon tissue sections were graded using a scoring system for inflammation. Combined scores from the proximal colon (PC), mid colon (MC) and distal colon (DC) segments are represented in the figure. Results were calculated by one-way ANOVA and Tukey's Multiple Comparison test. A significance is shown by $^{**}p < 0.01$, $^{***}p < 0.001$, data represents mean± SEM (n=6/ genotype).

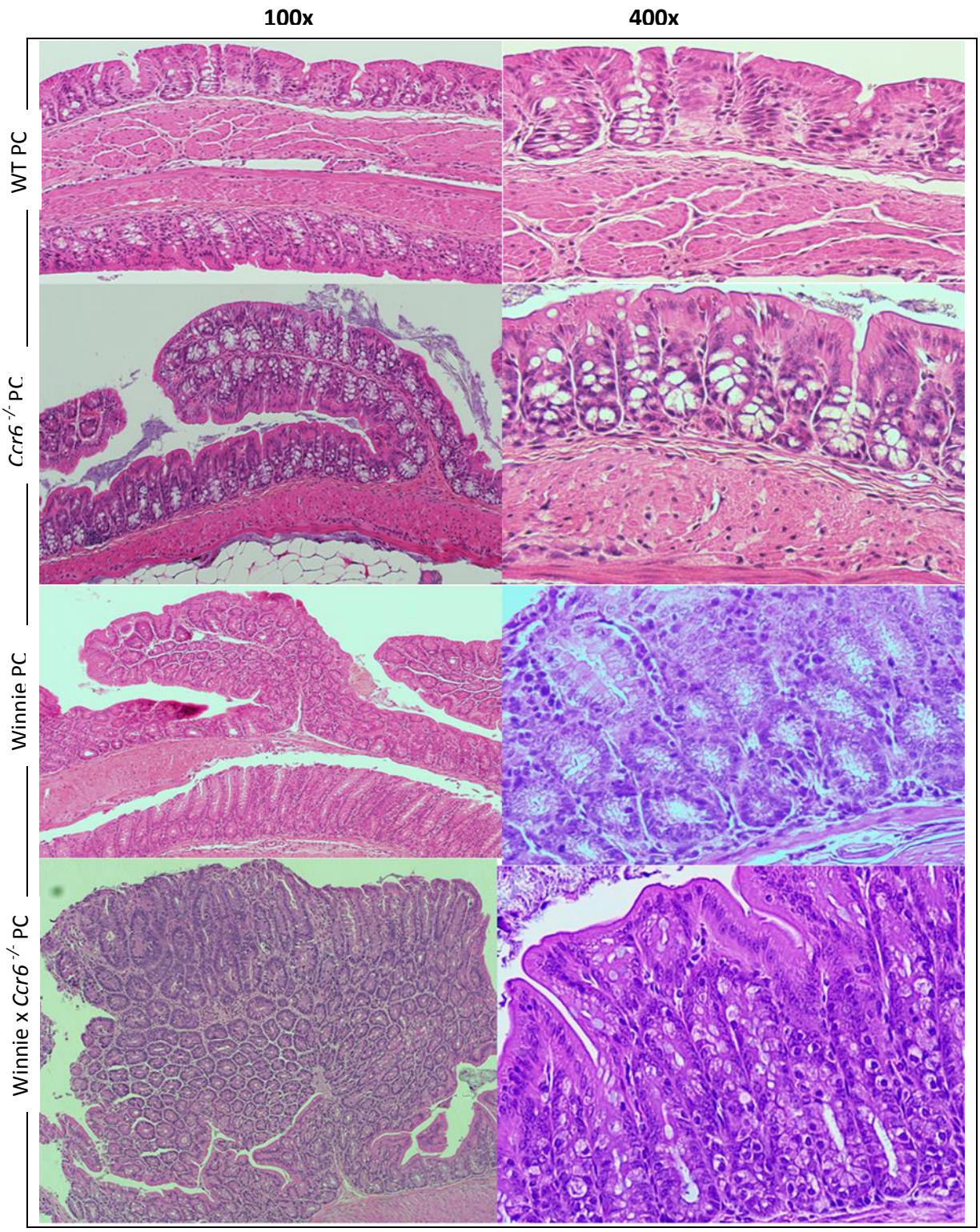


Figure 4.5: Comparative proximal colon histology from all genotypes.
Representative H&E sections of proximal colon displaying the differences in crypt architecture, presence or absence of goblet cells in the proximal colon between Wild-type, *Ccr6*$^{-/-}$, Winnie and Winnie x *Ccr6*$^{-/-}$ mice. Magnification 100x and 400x.

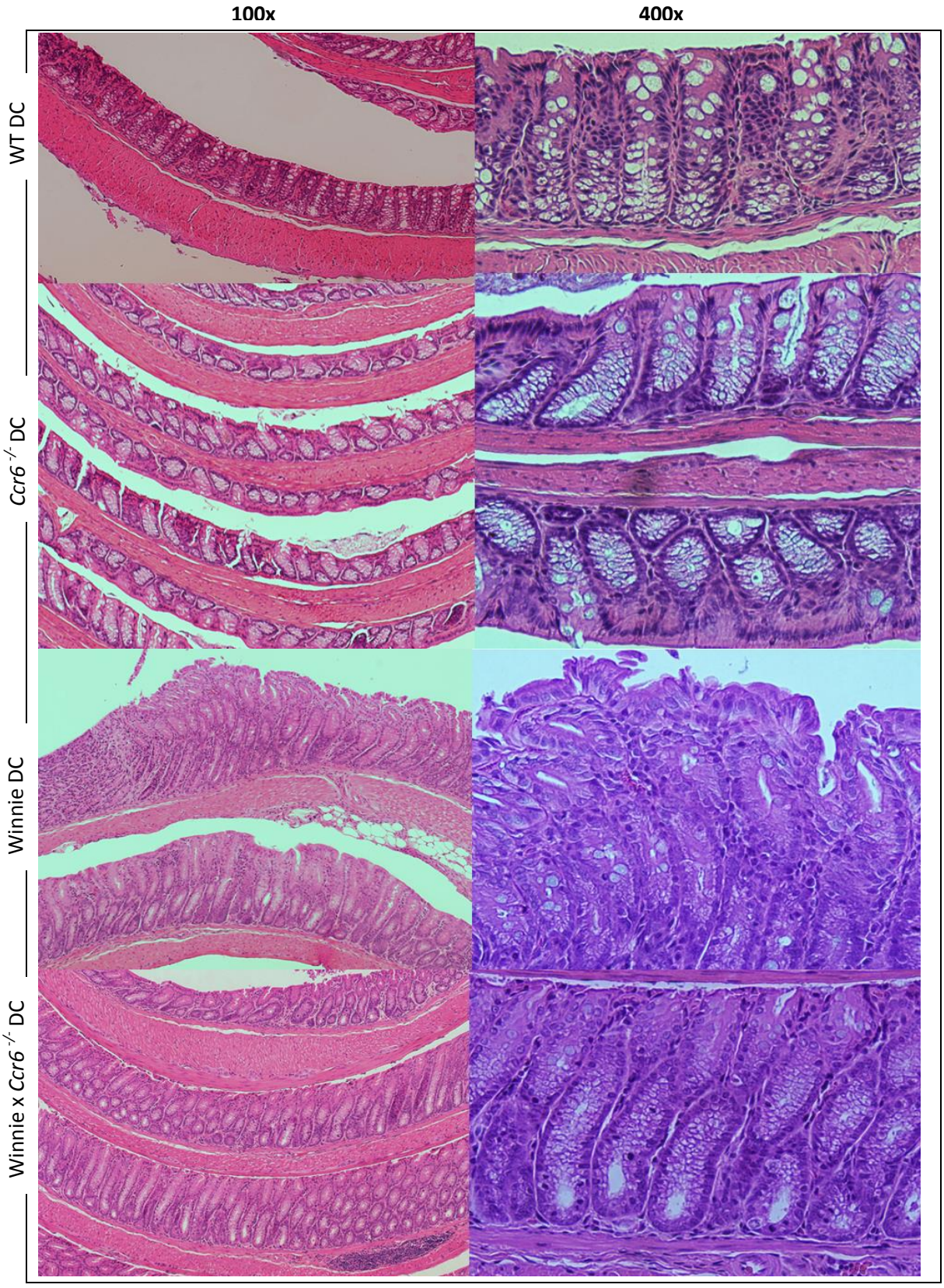


Figure 4.6: Comparative distal colon histology from all genotypes.
Representative H&E sections of distal colon presenting the differences in crypt architecture, presence or absence of goblet cells in the distal colon between Wild-type, *Ccr6*$^{-/-}$, Winnie and Winnie x *Ccr6*$^{-/-}$ mice. Magnification 100x and 400x.

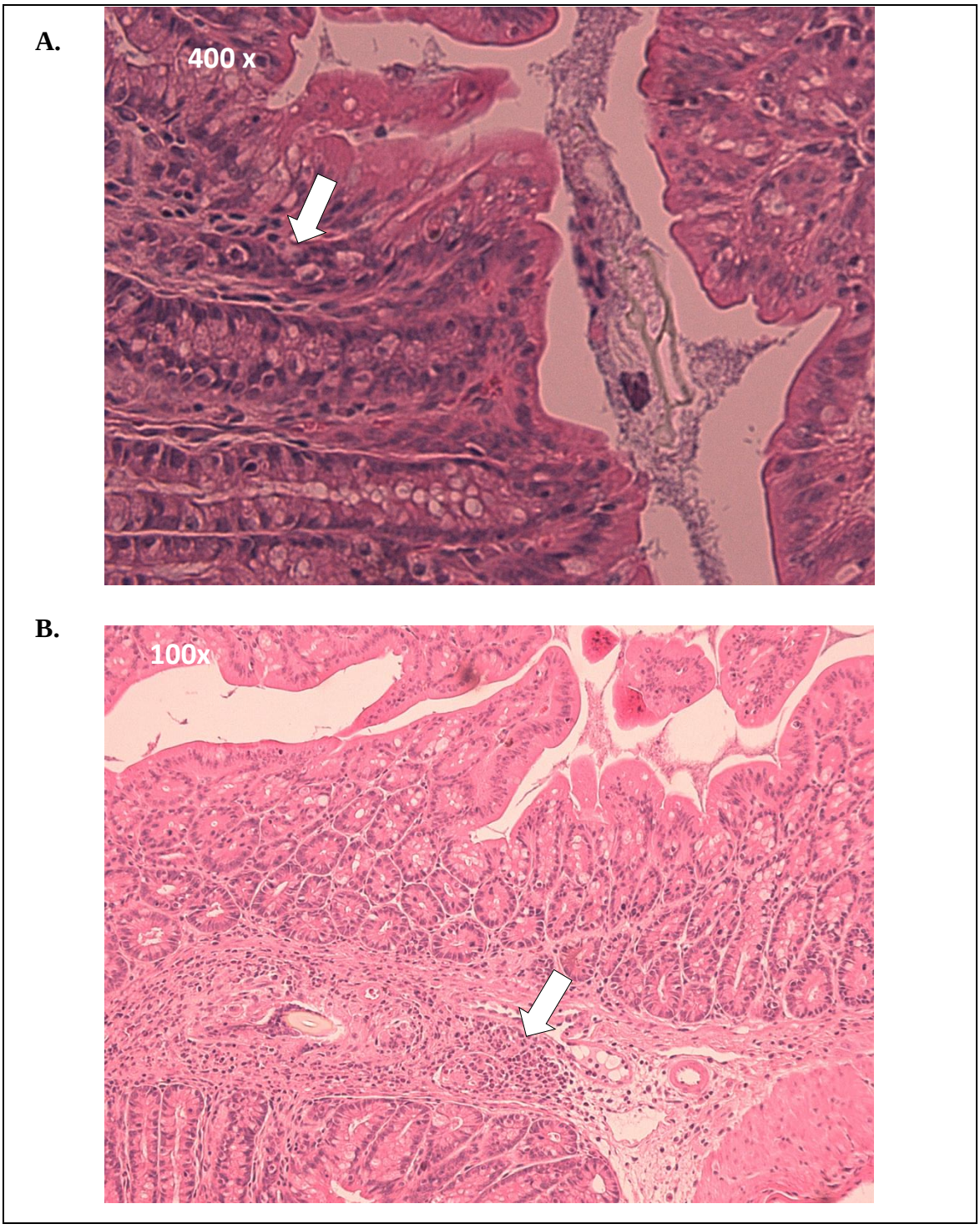


Figure 4.7: Increased inflammatory cell infiltration in PC of Winnie x *Ccr6*$^{-/-}$ mice.
A, B. Proximal colon of Winnie x *Ccr6*$^{-/-}$ showing inflammation in the submucosa and inflammatory cell infiltration in the lamina propria, goblet cell loss, crypt distortion. Magnification is 400x and 100x.

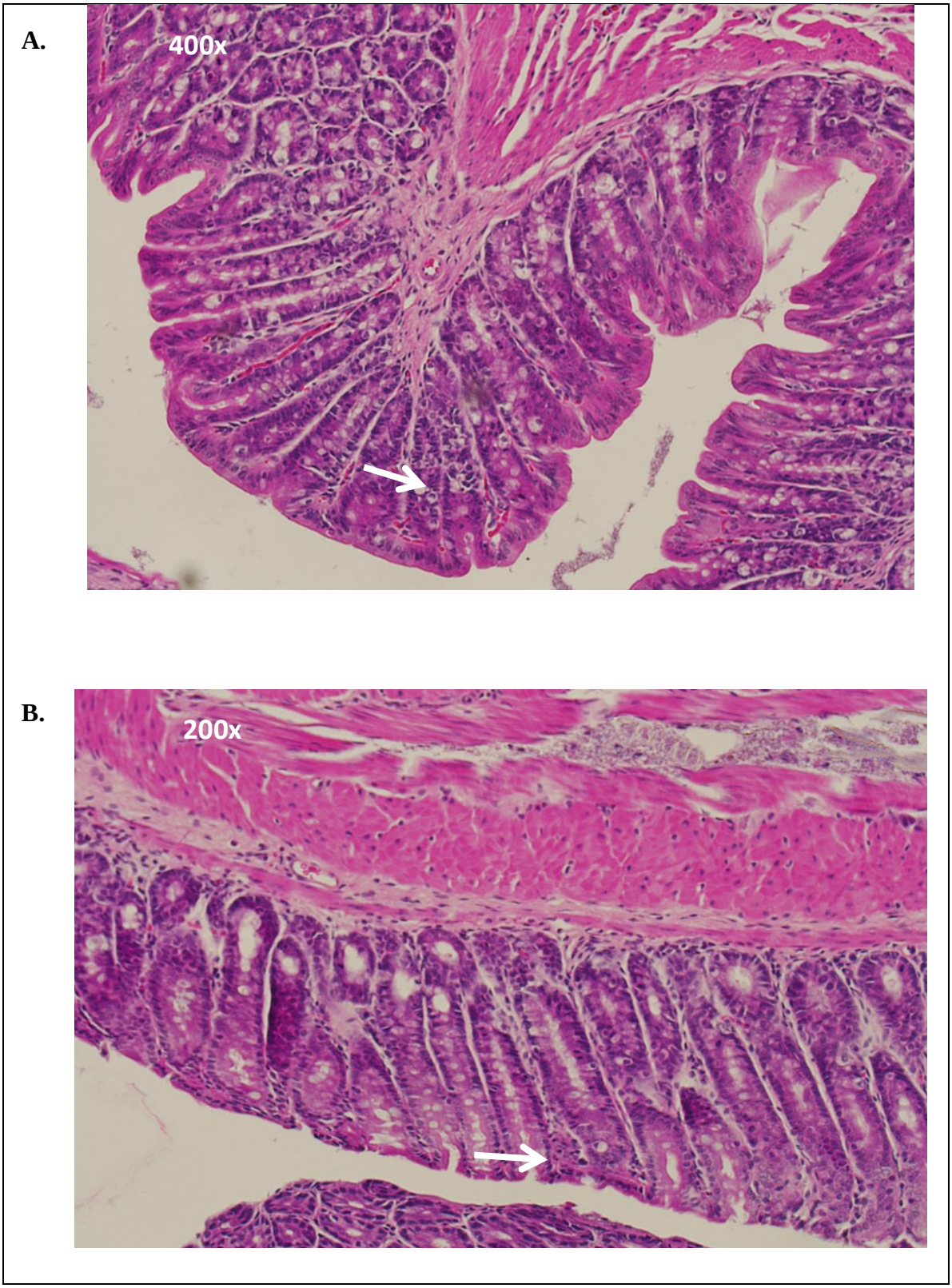


Figure 4.8: Increase in leucocytes in the lamina propria in the distal colon of Winnie. **A, B.** Prominent increase in leucocytes in the lamina propria, mild cryptitis, indicated by a mild distortion and elongation of the glands. Magnification 200x.

4.4 Discussion

Inflammation observed in the Winnie x *Ccr6*$^{-/-}$ mice progressively increased in severity from the distal to the proximal segment of the colon. In Winnie and Winnie x *Ccr6*$^{-/-}$ mice, the colon was observed to be swollen, and thickened relative to wild-type and *Ccr6*$^{-/-}$ mice. Unlike Winnie mice, the thickening of colon observed in Winnie x *Ccr6*$^{-/-}$ mice was most prominent in the caeco-colic region. Thickening of the colonic wall associated with colonic inflammation likely contributed to the increase in the colon weight of mice harbouring inflammation (Winnie and Winnie x *Ccr6*$^{-/-}$) [43,145]. Histopathological assessment of Winnie and Winnie x *Ccr6*$^{-/-}$ mice revealed all the classical symptoms of colitis such as crypt distortion, cryptitis, erosion, submucosal inflammation, and ulceration.

Hyperchromatic nuclei were observed in the proximal colon of Winnie x *Ccr6*$^{-/-}$ mice, suggesting the development of more severe pathological changes in this phenotype. The distribution of inflammation seen during histopathological grading in Winnie x *Ccr6*$^{-/-}$ emphasises an altered distribution of inflammatory leucocytes and cytokines compared to Winnie. The Winnie mouse model is remarkable in that colitis develops whilst the underlying immune system is normal [178]. Due to the presence of an unmodified immune system in the Winnie mouse strain, it is easier to interpret pathological changes resulting from genetic deletion of *Ccr6*. *Ccr6*$^{-/-}$ mice display underdeveloped intestinal Peyer's patches (PPs), an increased number of intra-epithelial lymphocyte (IEL) subpopulations and defective leukocyte homing to the intestinal mucosa [137,179].

The inflammation of the Winnie x *Ccr6*$^{-/-}$ model occurs primarily in the proximal colon so a defective leukocyte dynamic would likely be most pronounced here. In the Winnie x *Ccr6*$^{-/-}$ model we demonstrate spontaneous inflammation in the proximal colon to mid colon segment and mild inflammation in the distal colon of this phenotype.

In this study, a Winnie x *Ccr6*$^{-/-}$ mouse model was used to assess and analyse the development of the disease and their response to the inflammatory environment when compared with that of human colitis. In Winnie mice, due to the missense mutation, the effect is limited to intestinal secretory cells whereas the underlying immune system remains unaffected. Similar to other models of colitis, the lamina propria of the distal colon in Winnie mice displays a cytokine profile dominated by IL-17A and IFN-γ production from $CD4^+$ lymphocytes. The characteristic differences in the immune regulation of Winnie mice and *Ccr6*$^{-/-}$ mice could be the reason for the outcome of the inflammatory pattern observed in Winnie x *Ccr6*$^{-/-}$ mice. Although a large number of inflammatory infiltrates did not increase as age-progressed to 12 weeks in both Winnie and Winnie x *Ccr6*$^{-/-}$, the abundant inflammatory cell infiltration was mostly confined to the PC to MC segments in Winnie x *Ccr6*$^{-/-}$ mice. Neutrophils are recruited into the intestinal mucosa during an active inflammatory response which was observed in the Winnie x *Ccr6*$^{-/-}$ model.

The differential exacerbation demonstrated in the Winnie x *Ccr6*$^{-/-}$ mice colon might be due to the increased production of pro-inflammatory cytokines in the site of exacerbation [43] as Winnie mice have reportedly higher expression of pro-inflammatory cytokines such as IL-β, during intestinal inflammation [43]. Multiple factors are involved in the development of intestinal inflammation, specifically, the activation of NF-κB is said to be central during the development of inflammation. ER stress induces the activation of NF-

κB, and the transcription is increased as a result [43]. The pro-inflammatory cytokine IL-1β is elevated and increased in the intestines of both UC and CD patients and this is also observed with our Winnie colitis model [43,145]. The elevated expressions of T effector cells, in particular Th17 cells and associated cytokines such as IL-17 and IL-23 identified in Winnie mice demonstrates a dominant Th17 immune response resembling UC in humans [43,145]. Th17 effectors also promote neutrophil chemotaxis, and hence a mucosal neutrophilia could be expected in Winnie x *Ccr6*$^{-/-}$. CCR6 has been implicated in skewing the response in intestinal inflammation towards exacerbation or homeostasis based on the cytokine milieu. The IL-23/Th17 immune response demonstrated in Winnie mice is reportedly one of the major factors in the pathogenesis of IBD [180,181].

In Winnie x *Ccr6*$^{-/-}$ mice, the significant role of effector T cells in dictating the exacerbation of Winnie colitis and the activation of innate immune response in younger age Winnie mice were reported by Heazlewood et al. (2008). The differential inflammatory pattern observed in the colonic segments of Winnie vs Winnie x *Ccr6*$^{-/-}$ during histopathological inflammation demonstrates the crucial role of T cells in this phenotype. In Winnie mice, the balance between Th17 and Treg in sections of the colon determines the inflammatory phenotype and absence of *Ccr6* could possibly contribute to the change in the equation of Th17 and Treg differentially attributing to the outcomes observed in the Winnie x *Ccr6*$^{-/-}$ phenotype.

The pathology of Winnie x *Ccr6*$^{-/-}$ is in contrast to the pathology of Winnie colitis. Mild distal colitis develops in Winnie mice by 6 weeks of age and advances into a severe and chronic colitis as age progresses [131,182]. The clinical and histopathological features of Winnie colitis is similar to UC observed in human patients [43]. Characterisation of

inflammatory cell infiltrates can be conducted by using immunohistochemistry techniques. The use of colocalization assays would be advantageous in identifying Ccr6 and Ccl20 positive cells in the lamina propria of Winnie and Winnie x *Ccr6*$^{-/-}$ mice. This is a limitation of this study and must be considered in future studies.

In *Ccr6*$^{-/-}$ mice, colitis was induced by DSS and TNBS respectively. The histopathological results showed the absence of ulcers which suggested a reduction in the severity of intestinal pathology in the DSS-induced mouse model and susceptibility in the TNBS model which is otherwise resistant to intestinal inflammation [137]. As there is an increased existence of T cell subsets in the intestine of *Ccr6*$^{-/-}$ deficient mice when combined with the Winnie strain, the arising genotype would be expected to have a homeostatic or inflammatory phenotype [25,137].

Rectal prolapse in Winnie x *Ccr6*$^{-/-}$ mice was uncommon, with only a very small number of female Winnie x *Ccr6*$^{-/-}$ mice developing rectal prolapse at around 8–12 weeks. Winnie mice were reported to be prone to developing rectal prolapse associated with intestinal inflammation. However, only some of the Winnie mice have been reported to develop rectal prolapse and although there has been no specific age reported to date, it could occur at any time between 9–20 weeks [183].

4.5. Concluding remarks

In summary, the clinical assessment along with a histological examination of the Winnie x *Ccr6*$^{-/-}$ colon displayed exacerbation of inflammation in the PC to MC segment. In Winnie mice, the DC was inflamed and the PC remained mildly inflamed which was in correlation with previous findings. The *Ccr6*$^{-/-}$ mice colon and the WT mice colon were both non-inflamed and appeared to be normal. Overall, the Winnie x *Ccr6*$^{-/-}$ mouse model displayed a differential pattern of inflammation which could possibly be due to the recruitment of pro-inflammatory cytokines. Further characterisation of the Winnie x *Ccr6*$^{-/-}$ model based on molecular and immunological expression in the forthcoming chapters will provide a better understanding of this model.

Chapter 5

Immunological and Molecular Profile of Winnie x *Ccr6*$^{-/-}$ mice

5.1 Introduction

The immunological, molecular and cytokine profiles of Winnie x *Ccr6*$^{-/-}$ mice are described in this chapter. The cytokine environment and their responses are the main elements responsible for the initiation of inflammation in IBD. Intestinal immune responses initiate a cascade of events due to an inflammatory signal, and differentiated T and B cells along with the cytokine environment decide whether homeostasis or inflammation proceeds [184]. It is important to identify these immune mechanisms to establish a clear-cut definition of IBD pathogenesis in order to develop better treatment.

Chemokines and chemokine receptors are now the novel therapeutic targets [75] as they play a vital role in the immune system, in particular, they are involved in lymphocyte homing during homeostasis and inflammation [67,185]. Chemokine receptors organise the cells within lymphoid tissues and also the expression of other receptors in myeloid cells such as dendritic cells, and regulate their trafficking activities [186]. CCR6 is expressed on both Treg cells and Th17 cells; it serves as a distinct marker in differentiating both from the other types of T helper cells [103,109]. CCR6 is involved in B cell differentiation and humoral immunity. CCL20, the chemokine ligand for CCR6, is abundantly seen to be produced by colonic epithelial cells, dendritic cells and

subepithelial fibroblasts [94,187]. The presence of CCR6 on the surface of both anti-inflammatory Treg cells and pro-inflammatory T helper cells suggest dual functions, both enhancing inflammation and limiting its severity or extent. The interaction between chemokine and ligand in both the activation and resolution of colitis require further definition in order to understand the pathological skewing toward chronic inflammation. *Ccr6*$^{-/-}$ mice display underdeveloped Peyer's patches and due to this, the myeloid CD11b^{+} CD11c^{+} dendritic cell subset positioning is absent from the subepithelial dome. A diverse range of T cell subpopulations are expressed within the intestinal mucosa and are involved in intestinal immunity [137].

It is now established that *Ccr6* is important for recruitment and/or migration of both Treg cells and Th17 cells during inflammation and specifically during intestinal immune responses, and hence the *Ccr6*$^{-/-}$ mouse model was widely used to understand the role of *Ccr6* in intestinal inflammation [137]. Previous studies have reported that CCR6^{+} cells were more often identified in CD4^{+} T cell subsets than in CD8^{+} T cells subsets [187]. Of the CD8^{+} CCR6^{+} T cells, most were found to display a T effector memory (T_{EM}) phenotype [187]. *Ccr6* is highly expressed in immature dendritic cells and some studies using *Ccr6*$^{-/-}$ mice have demonstrated defects and the impaired dendritic cell presentation to CD4^{+} T cell populations in the Peyer's patches which emphasises the importance of the *CCR6* gene in mucosal immunity [99,187,188].

Genome-wide association studies reported that the IL-23/Th17 pathway plays a vital role in IBD pathogenesis and the genetic polymorphisms of Th17 signature genes *IL23R*, *IL-12B* (p40), *CCR6* and *STAT3* are reportedly associated with the initiation of both CD and UC [145]. In Winnie mice, dendritic cells (DCs) and macrophages, the antigen presenting cells (APCs), initiate an innate immune inflammatory response which is followed by

adaptive immune responses which progress toward a mixed Th1, Th2 and a more conspicuous Th17 mucosal immune response. The distal colon of Winnie mice featured increased levels of $CD11c^+$ APCs and activated dendritic cells DCs ($CD11c^+$ $MHC\text{-}11^{hi}$) which also assists in configuring a Th17-dominated mucosal immune response [43,145].

5.2 Aims/hypothesis

The aim of this present study was to identify the immunological, molecular and cytokine profile of Winnie x *Ccr6*$^{-/-}$ mice. Since Winnie x *Ccr6*$^{-/-}$ mice immunopathology needed to be described, we hypothesised that there will be an increased expression of Th17 signature genes and increased the population of $CD4^+IL\text{-}17^+$ T effector subsets in the absence of CCR6.

5.3 Results

5.3.1 Gene expression

A panel of inflammatory markers was chosen (Table 2.4) and gene expression assays were performed as described previously. Gene expression of the inflammatory markers was evaluated, normalising the data to *Eef2* (housekeeping gene) (Figure 5.1). Expression of pro-inflammatory *Il-1β* in the proximal colon segment of Winnie x *Ccr6*$^{-/-}$ mice was increased (P value < 0.01) compared to the proximal colon of Winnie mice.

In contrast, *Ccr6* deletion in Winnie mice did not discernibly alter *Il-6* levels in the proximal colon, whereas *Il-6* levels in the distal colon were increased (P value < 0.05) in Winnie x *Ccr6*$^{-/-}$ mice. The expression of *Il-6* varied in the distal colon compared to the proximal colon of Winnie x *Ccr6*$^{-/-}$ mice. A decrease (P-value < 0.001) in the expression levels of *Tgf-β1* was observed in both the proximal and distal colon of Winnie x *Ccr6*$^{-/-}$

mice when compared with the distal colon of Winnie mice. An increase (P-value < 0.001) of *Ccr6* levels were observed in the Winnie mice distal colon when compared to its proximal colon. In Winnie x *Ccr6*$^{-/-}$ mice, the *Ccr6* gene was not expressed as we expected which additionally affirmed the homozygous gene deletion, and a statistical significance (P value < 0.001) was reached relative to Winnie mice. *Ccl20* expression was increased (P value < 0.001) in the proximal colon of Winnie x *Ccr6*$^{-/-}$ mice and distal colon of Winnie mice. On the other hand, *Ccl20* levels were reduced (P-value < 0.001) in the proximal colon of Winnie mice and in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice.

Il-22 levels were significant (P-value < 0.001) in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice compared to its proximal colon and also when compared to the Winnie mice distal colon, *Il-22* had reduced (P-value < 0.001) in the distal colon. *Il-23a* expression was decreased (P-value < 0.001) in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice and in contrast significant (P-value < 0.001) levels were observed in the distal colon of Winnie mice. Foxp3 expression levels were varied (P-value < 0.01) in the proximal colon of Winnie x *Ccr6*$^{-/-}$ mice when compared to the significantly increased expression observed in the distal colonic segment of Winnie mice. *Il-10* expression was significantly reduced (P-value < 0.05) in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice, when compared to the Winnie mice distal colon and this relates to the milder inflammation, observed histologically in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice.

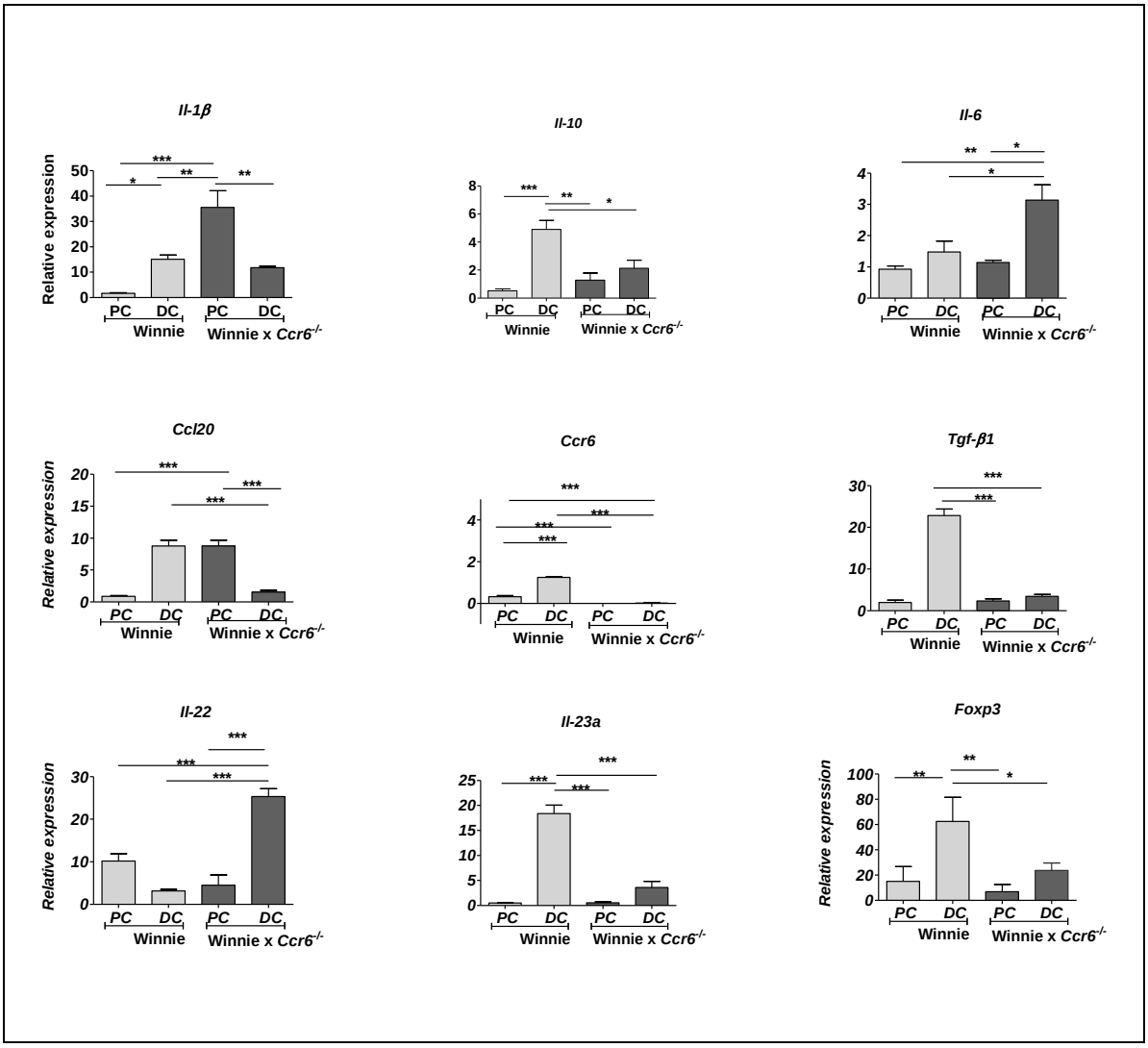
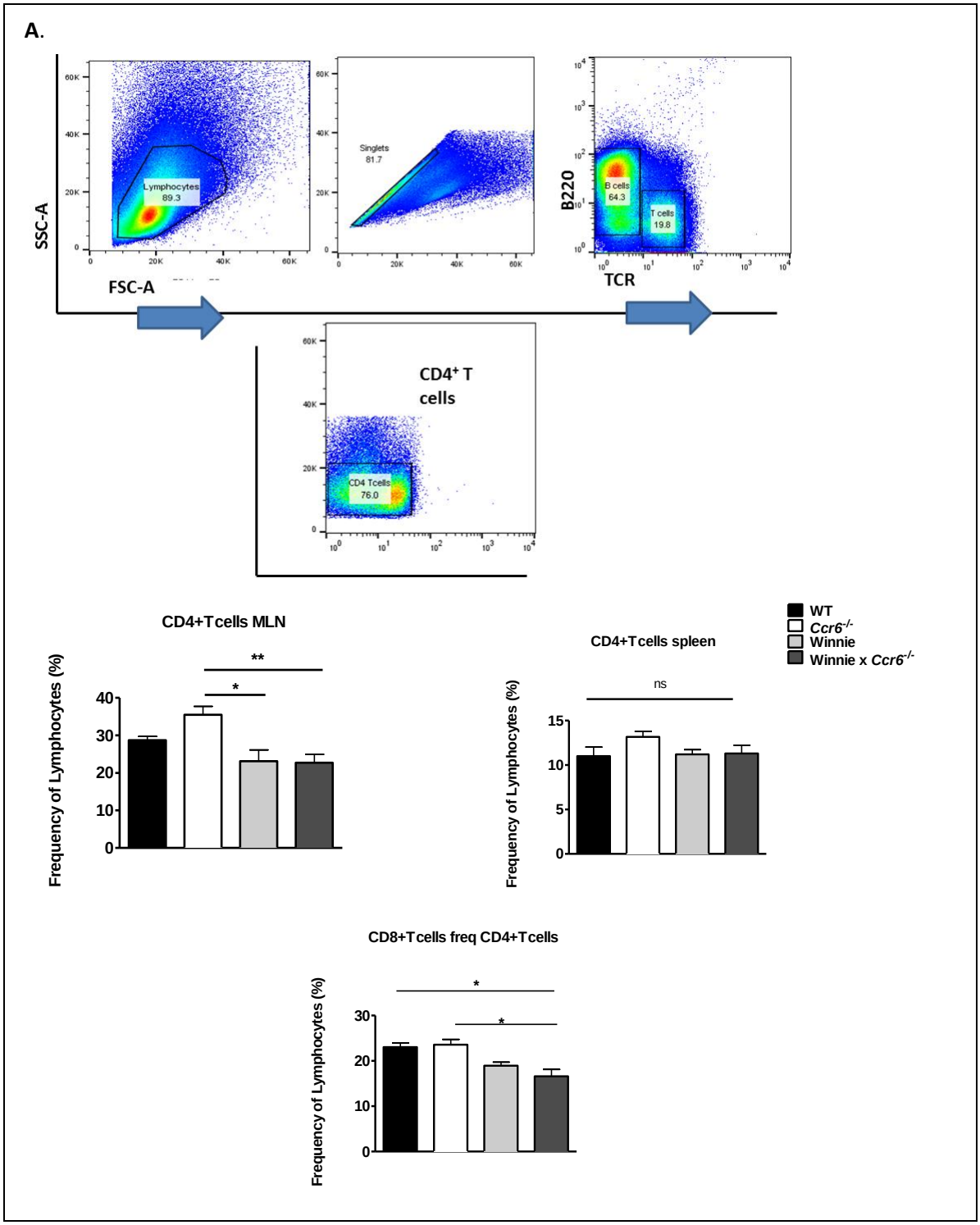


Figure 5.1 Relative gene expressions in the proximal and distal colon of Winnie and Winnie x *Ccr6*$^{-/-}$ mice.

The relative gene expression of inflammatory markers between proximal and distal colon samples of mice in all four genotypes was used and was amplified as 25ng of the template in duplicates and normalised to the housekeeping gene *Eef2* and quantified using $2^{-\Delta\Delta}$CT. Results were analysed using one-way ANOVA and Tukey's multiple comparison tests to compare the proximal and distal colon samples between the four genotypes. A significance is shown by $*p < 0.05$, $**p<0.01$, $***p<0.001$, data represents mean± SEM of (n=3/ PC and DC from each genotype).

5.3.2 Effect of CCR6 deficiency on $CD4^+$T cells in Winnie x *Ccr6*$^{-/-}$ mice

In the samples from WT, Winnie, *Ccr6*$^{-/-}$ and Winnie x *Ccr6*$^{-/-}$ mice the lymphocyte population was first identified and gated for and then the singlet populations were isolated. The B cells and T cells were identified by their markers B220 and TCR respectively before being identified and gated. $CD4^+$ T cells in both MLNs and spleen samples were then analysed on the flow cytometer and assessed. There was a statistically significant decrease (P value<0.01) of the $CD4^+$ T cell population in MLNs of Winnie x *Ccr6*$^{-/-}$ mice and Winnie mice (P <0.05) when compared with *Ccr6*$^{-/-}$ mice. WT spleen samples displayed no statistically significant differences in the size of the $CD4^+$ T cell population between all four genotypes (Figure 5.2A). The $CD4^+$ and $CD8^+$ T cell subpopulations in MLNs were reduced in Winnie x *Ccr6*$^{-/-}$ mice relative to WT, Winnie, and *Ccr6*$^{-/-}$ mice (P-value < 0.05). The activated IL-17^+FOXP3^- cell population in Winnie was relative to *Ccr6*$^{-/-}$ (which was around P <0.05) and it was statistically significant. On the other hand, an increasing trend of $IL17^+FOXP3^+$ T cells in MLNs of Winnie x *Ccr6*$^{-/-}$ was observed but regarded as not statistically significant.


A.
SSC-A
FSC-A
Lymphocytes
89.3
Singlets
81.7
B220
TCR
B cells
64.3
T cells
19.8
CD4+ T cells
CD4 Tcells
76.0
CD4+Tcells MLN
Frequency of Lymphocytes (%)
**
*
CD4+Tcells spleen
ns
WT
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Winnie
Winnie x Ccr6-/-
CD8+Tcells freq CD4+Tcells
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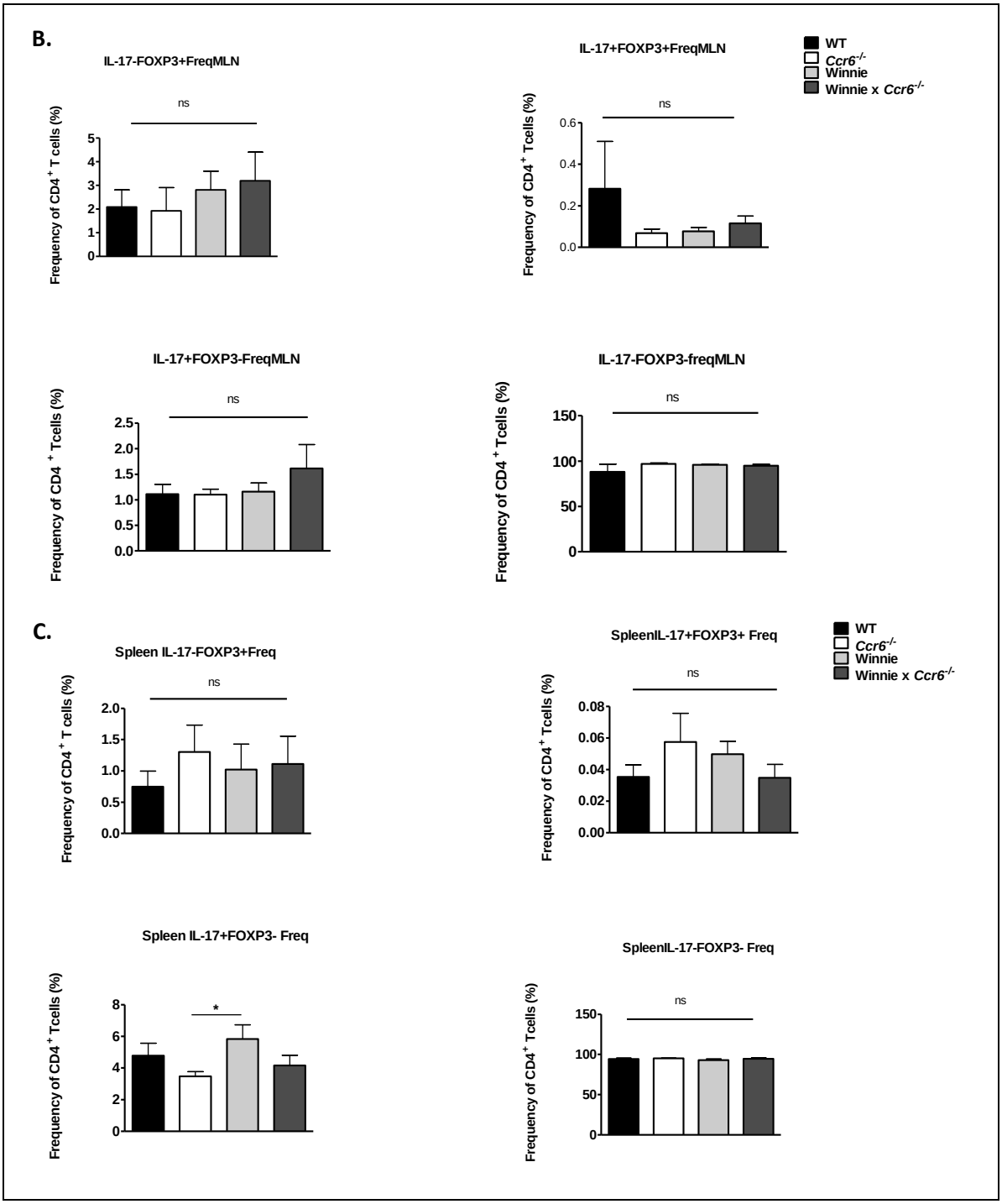

Figure 5.2 Quantification of $CD4^+$ T and $CD8^+$ T cells in Winnie x *Ccr6*$^{-/-}$

A. The frequency of $CD4^+$ T cells to $CD8^+$ T cell population was quantified in both spleen and MLNs.

B. Mesenteric lymph nodes (MLNs) were retrieved along with spleen from all four genotypes and stimulated and stained before being analysed by flow cytometry. The lymphocyte populations (T and B cells) were identified and $CD4^+$ T cells were then identified. $IL17^+FOXP3^+$, $IL17^-FOXP3^-$, $IL17^-FOXP3^+$, $IL17^+FOXP3^-$ cells were identified and quantified from the $CD4^+$ T cell population. The frequency of lymphocytes was quantified from MLNs.

C. Splenocytes were isolated, lysed, processed, stimulated and intracellular stained to identify the $CD4^+$T cells. IL17 and FOXP3 populations were identified and quantified from $CD4^+$Tcell population.
Figure 5.2 A/B/C represents data generated from 2 independent experiments (n=3/genotype). One-way ANOVA & Dunn's multiple comparison tests, *****significant at $p < 0.05$.

5.3.3 Assessment of CCR6 expression profile in Winnie x *Ccr6*$^{-/-}$ mice

MLNs and splenocytes were harvested from WT, *Ccr6*$^{-/-}$, Winnie and Winnie x *Ccr6*$^{-/-}$ mice and were stimulated *in vitro* for 4 hours prior to fixation, permeabilization and intracellular staining. The samples were subsequently analysed by flow cytometry and there was found to be no statistical significance (Figure 5.3A) between the four groups. Data revealed the expression pattern of $Foxp3^+$ T cells and IL-$17A^+$ T cell population in Winnie x *Ccr6*$^{-/-}$ mice. The MLNs from Winnie x *Ccr6*$^{-/-}$ showed a decreased (P-value <0.05) population of $CD4^+$ $IL17^+$ $FOXP3^+$ T cells expressing Ccr6 relative to Winnie (Figure 5.3B). There was a statistically significant reduction observed in the $Ccr6^+FOXP3^-$ Treg cells and $Ccr6^+IL17^+$ Th17 (P-value <0.05) in Winnie and *Ccr6*$^{-/-}$ MLNs.

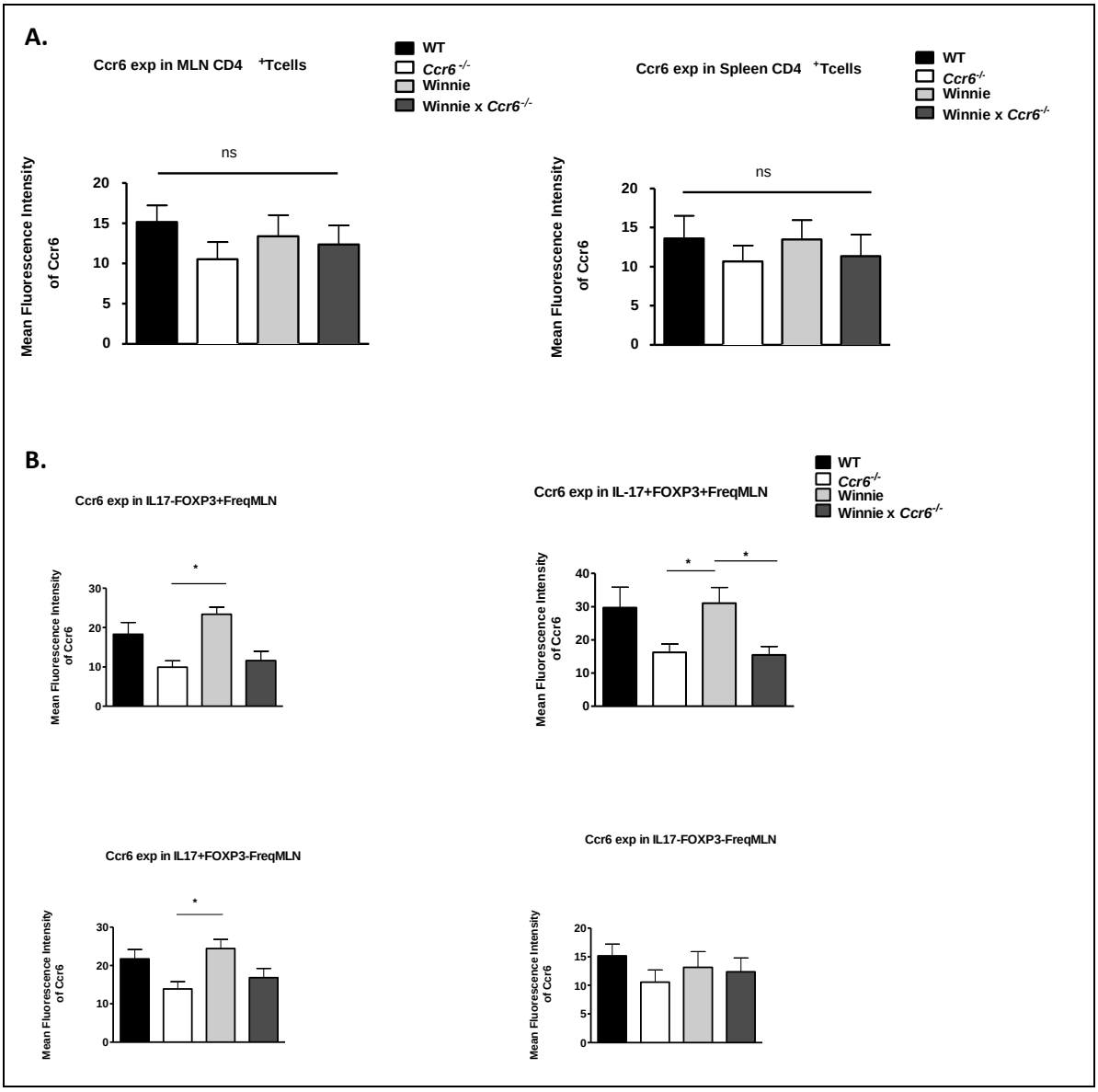

Figure 5.3: Quantification of Ccr6 expression in CD4$^+$ T cell subsets in MLNs.
A. The Ccr6 expression on CD4$^+$ T cells from MLNs and splenocytes of WT, *Ccr6*$^{-/-}$, Winnie and Winnie x *Ccr6*$^{-/-}$ mice, data represents no significance between the four groups (n=3/genotype), data pooled from 2 independent experiments.
B. MLNs removed from WT, *Ccr6*$^{-/-}$, Winnie and Winnie x *Ccr6*$^{-/-}$ mice were stimulated *in vitro* for 4 hours prior to fixation, permeabilization and intracellular staining. The MLNs were intracellularly stained using BD fix perm kit. Mean fluorescent intensity (MFI) of Ccr6 was quantified from 2 independent experiments (n=3/gentoype). One-way ANOVA, Dunn's multiple comparison tests,*****significant at p <0.05.

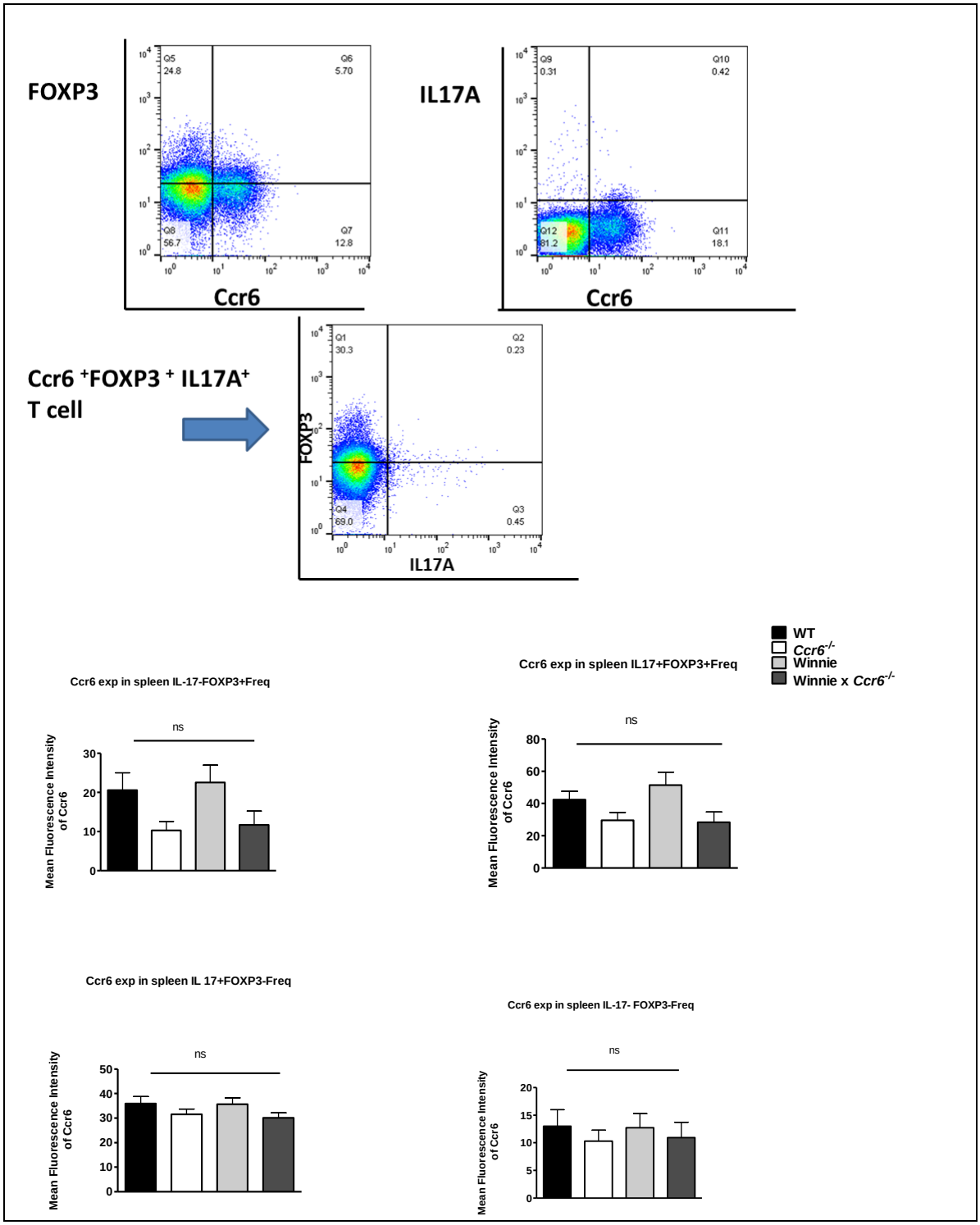

Figure 5.4: Quantification of Ccr6 expression in splenocytes.
Splenocytes removed from WT, *Ccr6*$^{-/-}$, Winnie and Winnie x *Ccr6*$^{-/-}$ mice were stimulated *in vitro* for 4 hours prior to fixation, permeabilization and intracellular staining. Mean fluorescent intensity (MFI) of CCR6 was quantified from 2 independent experiments (n=3/genotype). One-way ANOVA, Dunn's multiple comparison tests, *****significant at $p < 0.05$.

5.3.4 Cytokine profile and T cell expansion assay of Winnie x *Ccr6*$^{-/-}$ mice

Cytokine assays were performed on colonic explant cultures and the results revealed that the proximal colon of Winnie x *Ccr6*$^{-/-}$ mice showed an increased expression of IL-1β when compared to the Winnie mice distal colon. Also, the expression of IFN-γ was reduced in the proximal colon and increased in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice. In Winnie mice, the proximal colon showed an increased (P value < 0.01) level of IFN-γ and a decreased level in the distal colon. Winnie x *Ccr6*$^{-/-}$ mice had lower levels of IL-17A in the proximal colon but an increase in the distal colon explant sample was observed, whereas Winnie mice displayed a decreased expression of IL-17A in both distal and proximal colonic explant cultures. These results were statistically non-significant (Figure 5.5)**.**

A T cell expansion assay was performed *in vitro*, and the MLNs were retrieved and cells were isolated from WT, Winnie x *Ccr6*$^{-/-}$, Winnie and *Ccr6*$^{-/-}$ mice and were cultured at a cell concentration of 2 x 10^6 / mL, activated with anti-CD3 and anti-CD28 for 24 hours and IL-17A cytokine levels were significantly increased relative to *Ccr6*$^{-/-}$ (shown at P <0.05) (Figure 5.6).

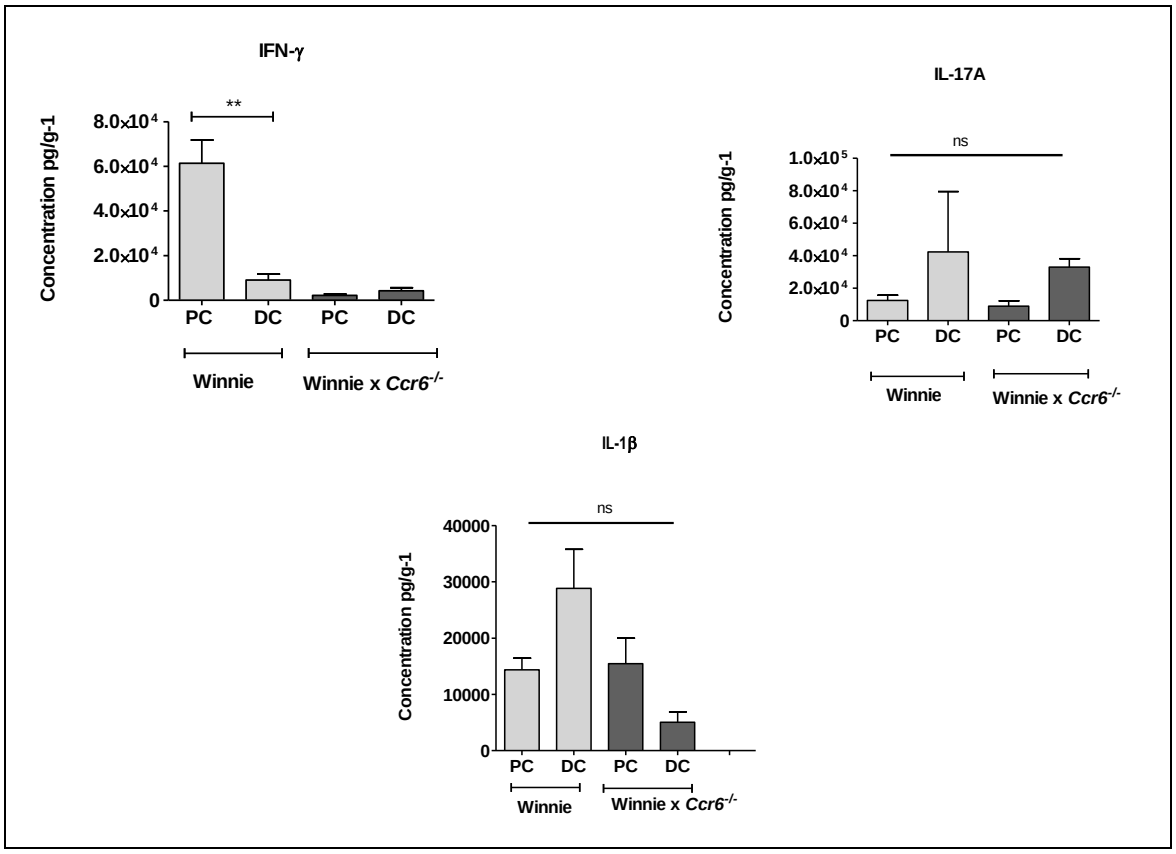

Figure 5.5 Increased IL-1β in colonic explant culture in Winnie x *Ccr6*$^{-/-}$ mice.
The colonic explants from Winnie and Winnie x *Ccr6*$^{-/-}$ proximal and distal colon samples were cultured for 24 hours. The supernatants were assessed for cytokine levels using Milliplex assay kit. Cytokine levels in the supernatants were normalised to tissue weight and obtained pg/ml of cytokines/gm of tissue. The figure represents data from 2 experiments pooled together; median fluorescence was calculated and the results did not reach a statistical significance. One-way ANOVA, Dunn's multiple comparison tests, **significant at $p < 0.01$.

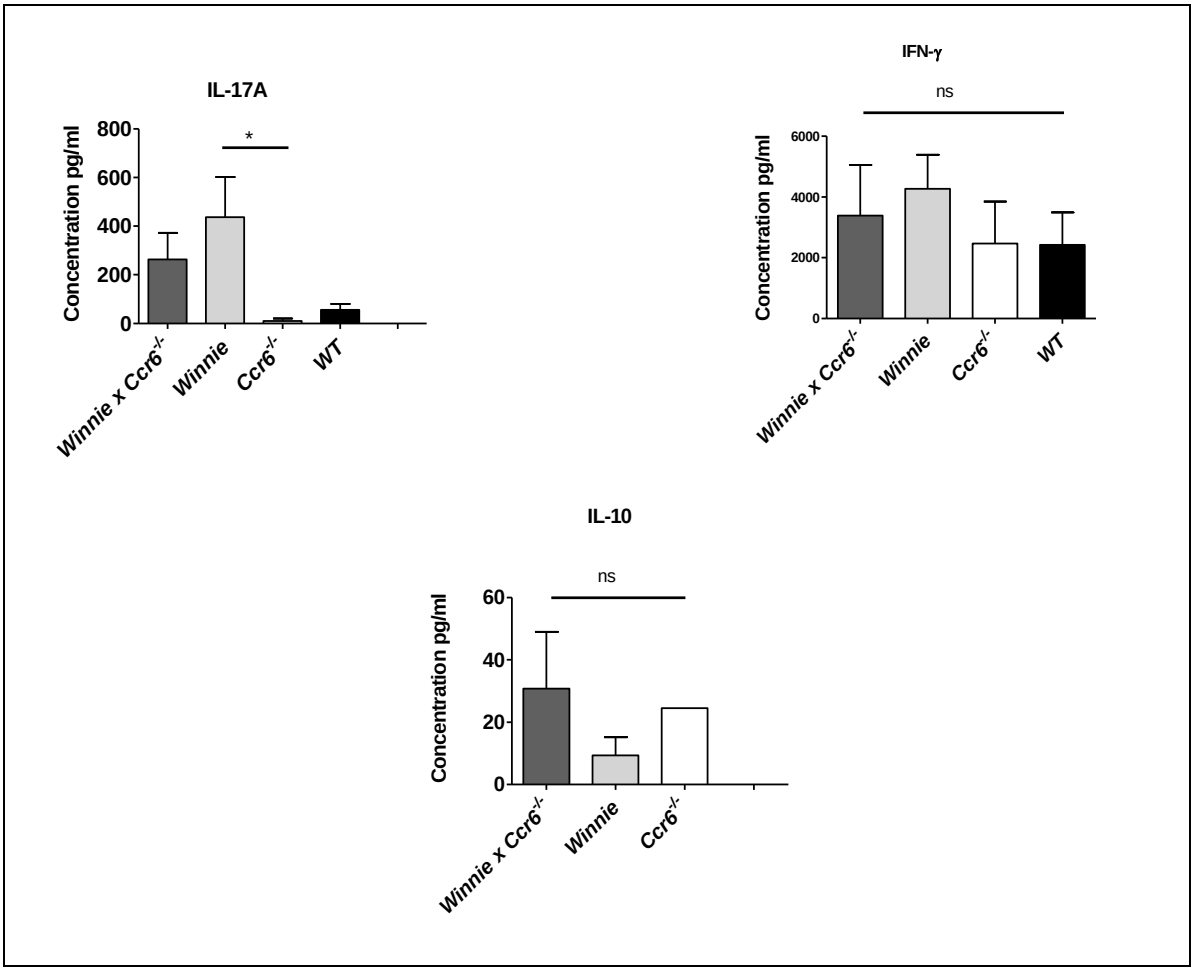

Figure 5.6 Alterations in activated T cells in Winnie x *Ccr6*$^{-/-}$ mice.
T cell expansion in vitro T cell stimulation assay, a concentration of $2x10^6$ / ml cells was cultured with anti-CD3 and anti-CD28 for 24hours. The figure represents data (n= 5 / genotype) from IFN-γ, IL-17A, IL-10 producing T cells which were analysed by one-way ANOVA (non-parametric), Dunn's multiple comparison tests, *****significant at $p < 0.05$.

5.4 Discussion

The immunological, molecular and cytokine profile of Winnie x *Ccr6*$^{-/-}$ mice was analysed in this chapter to understand the underlying immunopathology. The first step was to analyse the gene expression in order to identify and understand the differential pattern of colitis observed in Winnie x *Ccr6*$^{-/-}$ mice. A panel of inflammatory markers genes, namely *Il-1β*, *Il-10*, *Il-23*, *Il-22*, *Il-6*, *Tgfβ1*, *Ccr6* and *Ccl20*, were utilised to

understand the course of immune-mediated events and the subsequent immune responses and chemokine patterns involved, resulting in colitis in the differential pattern in the histopathological evaluation process. The immunopathology in Winnie mice is well established and features a possible Th17-mediated immune response to an intestinal epithelial dysregulation driven by Muc2 mutation. Genetic ablation of the *Ccr6* gene in Winnie mice resulted in a marked increase in the abundance of *Il-1β* and Ccl20 mRNA transcript in the proximal colon.

Cytokine assays confirmed a corresponding increase in total IL-1β in the proximal colonic explant cultures. Increased production of the pro-inflammatory cytokine IL-1β in the proximal colon of Winnie x *Ccr6*$^{-/-}$ mice was consistent with the pronounced exacerbation of inflammation observed during the clinical and histological evaluation. Significantly increased expression of IL-1β cytokine in cultured colonic explants has been reported in Winnie mice when compared with WT mice [145]. Increased expression of IL-1β in our mouse model is consistent with increases also reported in both UC and CD in humans [145]. Interestingly, Ccl20 had increased abnormally in the inflamed proximal colon but actually seemed to be reduced in the distal colon relative to Winnie mice, suggesting that the bulk of Ccl20 produced in the inflamed distal colon of Winnie mice is produced by CCR6 cells or is dependent on their factors, such as IL-17 and TNF-α [90].

Expression of *Il-6* and *Il-22* were both increased in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice whereas *Foxp3*, *Il-10* and *Il-23a* decreased in the same tissue. IL-22 is a unique cytokine produced by immune cells and this cytokine plays a vital part in host defence mechanisms at mucosal surfaces and is also involved in tissue restoration. IL-22 is beneficial to the host in various infectious and inflammatory conditions, although it depends on the target tissue to be pathogenic. Due to its inherent pro-inflammatory traits,

when exerted with other pro-inflammatory cytokines, IL-17 is proven to play a major role in inflammatory diseases [189-191]. IL-17 producing T cells were reduced when compared to Winnie mice in our findings; however, *Ccr6* deletion is known to alter the frequency of IL-17A^{+} T cells [192].

Production of IL-22 by Th17 cells is greater than the amounts produced by undifferentiated Th0 cells or Th1 cells [189]. It has been discovered that IL-6, by itself or along with TGF-β, has been reported to be essential for the induction of IL-17 in naïve T cells [193]. TGF-β has reportedly been shown to suppress production of IL-22 in a dose-dependent manner [189,193,194]. TGF-β is involved in the Th17 differentiation in the presence of IL-6 and hence increased expression of inflammation was observed in our current study. This is probably due to its anti-inflammatory trait which could possibly result in a reduction of inflammation [195].

Therefore the increased expression of IL-22 observed in Winnie x *Ccr6*$^{-/-}$ mice are consistent with the predicted Th17-mediated mucosal response in this phenotype. As previously reported, the high expression of IL-6 in Winnie mice colonic explant cultures, along with IL-12/ 23p40, indicated a Th17-mediated mucosal response [145]. The signature cytokines of Th17 expression observed in Winnie x *Ccr6*$^{-/-}$ mice indicate that the Th17 mucosal response could possibly characterise the proximal [189] colitis perceived in this phenotype, although IL-17 is not increased and IL-23A seems to be unaffected by the absence of *Ccr6* (Figure 5.7). IL-23/Th17 dominated mucosal immune response in Winnie reported in the literature was not concurrent with our findings in Winnie x *Ccr6*$^{-/-}$ proximal colitis [145,180]. The alterations in cytokine expression in Winnie x *Ccr6*$^{-/-}$ could possibly be due to varied animal housing conditions [43]. The

findings need to be further dissected to affirm Th17 dominated mucosal response in Winnie x *Ccr6*$^{-/-}$.

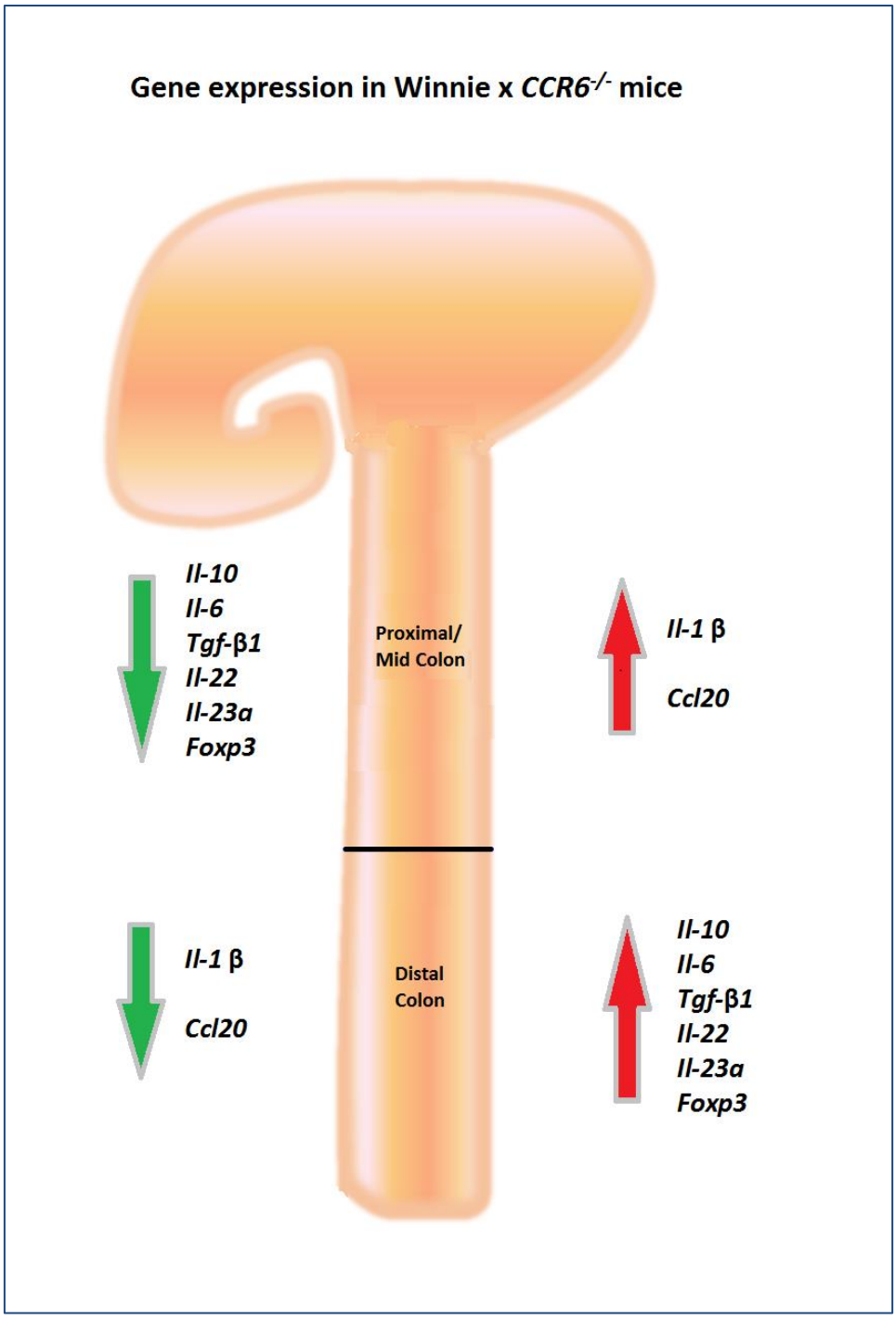

Figure 5.7: Genetic ablation of *Ccr6* increases IL-1β and IL-10 in the proximal and distal colon of Winnie x *Ccr6*$^{-/-}$ mice.

The immunological parameters of Winnie x *Ccr6*$^{-/-}$ mice were assessed and CD4^{+} T cell subsets following stimulation from MLNs and spleen were analysed. Treg cells are known to be involved in gut immune tolerance and proceed to offer tolerogenic control in murine colitis models, where the accumulation of FOXP3^{+} Treg cells are defined in various chronic inflammatory disorders. However, in our study, the CD4^{+} T cells were identified and specifically Ccr6^{+} FOXP3^{+} T cells and Ccr6^{+} IL17A^{+} Th17 cells were identified. FOXP3 cells were decreased which was in line with qPCR findings, the ratio of IL-17A^{+} FOXP3^{+}T cells in the absence of *Ccr6* deletion needs to be studied further.

Reduction of CD4^{+}FOXP3^{+} Tregs observed in our findings affirms the importance of innate immunity and the immune cells involved. It was previously suggested that in Winnie mice the balance between Th17 and Treg cells could possibly determine the inflammatory phenotype and that the absence of *Ccr6* in Winnie x *Ccr6*$^{-/-}$ mice possibly facilitates a variance of pattern in colitis severity, which was observed.

Either a reduction of CD4^{+} FOXP3^{+} Treg cells or suppression of efficient immunosuppression in Winnie x *Ccr6*$^{-/-}$ mice may be crucial in down-regulating the inflammation of the colon [145]. Increased expression of *Ccl20* in Winnie x *Ccr6*$^{-/-}$ mice corroborates the expression of both *Ccr6* and *Ccl20* in experimental murine colitis models as established in previous studies [25,95]. The follicle-associated epithelium is an important CCL20 producer for maintaining the GALT [98]. *Ccr6* possibly takes on a regulatory role as it has been previously reported and contributes to the regulation of IL-10 production in a steady state, and selectively migrates to the colon while the inflammation proceeds [95]. Hence, this study concludes that possibly CCR6 expression by Treg cells could potentially play a role in conferring Treg cells the ability to mediate immune regulation via anti-inflammatory cytokines such as IL-10 to the inflamed distal

colon. Novel colon homing, IL-10 derived from $Ccr6^{+}$ Tregs decreases the severity of distal colitis in Winnie x *Ccr6*$^{-/-}$ mice [95,144,145,196].

Previous studies have reported that IL-10 is preferentially induced by lamina propria macrophages whereas DCs facilitate the differentiation of Th17 cells [195]. In a similar fashion, we presume that the IL-10 expression found in the distal colon of our mouse model Winnie x *Ccr6*$^{-/-}$ would possibly promote immune-modulatory function, restricting the inflammation in this colonic segment in particular and thereby ameliorating the inflammation.

The well-established immune-modulatory functions of IL-10 and its associated signalling modules could serve as mediation for disease regulation especially in the gut as this environment favours the production of IL-10 and provides tolerance to commensal microbiota, whereas inflammatory responses are modulated in peripheral tissues during infections [195,196].

Earlier studies of Winnie mice reportedly identified that due to ER stress the DCs and epithelial cells activate and discharge cytokines such as IL-1β, IL-23 and IL-6, and are involved in mediating Th17 and Th1 immune responses subsequently followed by the release of IFN-γ and IL-17, and this is consistent with our current findings [145].

IL-10 and TGF-β1 generating Treg cells become overwhelmed by the pro-inflammatory responses and these proceedings initiate a sequence of intestinal inflammation. Increased expression of *Il-1b* in Winnie x *Ccr6*$^{-/-}$ mice is similar to that of the Winnie mice distal colon [175]. DCs produce *Il-6* and *Il-23a* with a Th17 favouring chemokine milieu when stimulated, and an increased mRNA expression of *Tgfβ1*, *Il17* and *Ccr6* in Winnie mice portrayed a Th17 immune response. In Winnie x *Ccr6*$^{-/-}$ mice, the colitis demonstrated is thought to be a multi-cytokine mediated inflammation and proves to be consistent with

the colitis observed in Winnie mice except for the differential pattern conferred by the genetic ablation of *Ccr6*.

5.5 Concluding remarks

The immunological, molecular and cytokine profile of Winnie x *Ccr6*$^{-/-}$ mice was associated with the pattern observed with the clinical and histological findings. Immuno-phenotyping of stimulated colonic lymphocytes indicated a reduction in the FOXP3^{+} Tregs and increased Th17 cells in the MLNs, but the splenic populations remained unaffected in the absence of *Ccr6*, which is in contrast to current literature. Cytokine assays and gene expression assays affirmed and revealed increased expression of pro-inflammatory cytokine IL-1β which was correlated with the clinical and histopathological findings. An increased level of IL-10 expression in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice was also observed, and this suggests IL-10 mediated amelioration of colitis which would otherwise exist in the Winnie mice as reported by Eri et al. and Wang et al. [145,180]. In comparison to Winnie colitis, all the above findings conclude that the spontaneous colitis demonstrated in Winnie x *Ccr6*$^{-/-}$ mice in the absence of *Ccr6*, displayed an exacerbated proximal colitis and ameliorated distal colitis in a spontaneous manner.

Chapter 6

General Discussion

IBD is a multifactorial, immune-mediated disorder, characterised by intestinal inflammation where CD and UC are the two subtypes. Genome-wide association studies have linked a number of significant loci involved in contributing to the disease progression of both CD and UC. *Ccr6* is one of the important loci implicated in IBD and it has widely been accepted that *Ccr6* is involved in both immune tolerance and inflammation in the gut. CCR6, along with its ligand CCL20, is involved in mucosal adaptive immunity and directing T cells to the inflammation site. There exists a knowledge gap between the extent and the balance of the relevant influences that *Ccr6* imparts to the immune system during inflammation/homoeostasis.

To facilitate the understanding of the pathophysiological course of IBD and to assess the novel therapeutic strategies related to exact mechanisms, knockout mouse models were developed. The availability of knockout and transgenic mouse models outlining the exact pathways associated with the development of both CD and UC is useful in the development and evaluation of therapy for IBD (Table 6.1). Currently available animal models are either chemically induced or genetically engineered models representing either CD or UC for determining the underlying pathogenesis of the disease. This will assist in designing experiments for the development of various therapeutic approaches in IBD.

Table 6.1 IBD Mouse models specific to CD and UC involved in therapeutic research

Mode of induction	CD	UC
Chemical	• TNBS • Indomethacin	• DSS • TNBS • Oxazolone • Acetic acid • Sulfhydryl inhibitors
Transgene/knockout	• SAMP1/YitFc • N-cadherin dominant negative mutant • $TNF^{\Delta ARE}$	• *TCRα*$^{-/-}$ • *WASP-/-* • *Mdr1a*$^{-/-}$ • *IL-2*$^{-/-}$ • *Gαi2*$^{-/-}$ • *IL-7 Tg* • TRUC(Tbet$^{-/-}$XRag2$^{-/-}$) • TGFβRIIDN • C3H/HeJBir
Adoptive transfer		

Adapted from references [197,198]

(Table 6.1) Abbreviations: ΔARE, a deletion mutant in AU-rich elements; DSS, dextran sulfate sodium; Gαi2, guanine nucleotide-binding protein G(i) subunit α-2; IL, interleukin; Mdr1a, multiple drug resistance 1a; TCR, T-cell receptor; Tg, transgenic; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNF, tumor necrosis factor; TRUC, T-bet(−/−)RAG2(−/−) ulcerative colitis; WASP, wiskott–Aldrich syndrome protein; TGF, transforming growth factor.

The chemically induced TNBS mouse model of colitis reportedly did not reiterate the aetiopathogenesis of CD although the relevant involvement of *NOD2*, a susceptibility gene in CD, was related to the disease development in this model [199]. However, the indomethacin model displayed extensive mucosal injury and mesenteric margins were identified as susceptible to CD [197,200]. In the commonly used DSS model for UC, colitis is indirectly caused by injury to the epithelium and elicits inflammation. Several doses of DSS need to be administered to depict chronic changes as seen in human patients and increased variability in disease severity are seen in this model.

Therapeutic interventions such as gene/cell therapy, chemical and microbial therapy, have been effective in this model [119,197,201]. The TNBS-induced colitis model displays a Th2 immune response and closely mimics CD. The oxazolone-induced colitis model also displays a Th2 immune response and showcases all the classic symptoms of UC. Therapeutic interventions such as Simvastatin have been tested on these two mouse models although the outcomes were opposing [197,202]. Acetic acid imparts injury to the mucosal epithelium, thereby inducing colitis resembling UC. Reports suggest that the acetic-acid model is suitable for drug-efficacy testing and compounds targeting reactive oxidative species and confer amelioration. However, this mode of induction needs to be tested at different time points, as the one elicited prior to 24hrs is not immunological [202].

Transgenic/knockout models such as SAMP1/YitFc and $TNF^{\Delta ARE}$ are animal models resembling CD, and *IL-7 Tg* mice, $TCR\alpha^{-/-}$, $WASP^{-/-}$, $Mdr1a^{-/-}$ and $IL\text{-}2^{-/-}$ are some examples of animal models for UC [197,198]. These genetically engineered models are involved in drug efficacy assessments. SAMP1/YitFc and $TNF^{\Delta\Delta RE}$ are two spontaneously occurring mouse models of CD, demonstrating a remarkable similarity to the human condition by disease manifestation, histological presentation and the response to current treatments [203,204]. These two murine models are used for the development of novel therapeutic strategies for chronic ileitis, as it reportedly responds well to conventional anti-inflammatory and immunosuppressive treatments [203].

N-cadherin-dominant negative mutant mice develop CD-like transmural inflammation in the jejunum [133,205]. Cadherins are involved in development and facilitate cell adhesion. These mice are utilised to identify their role in intestinal inflammation and intestinal neoplasia [205].

TCRα$^{-/-}$ is a Th2-mediated spontaneous chronic colitis model which closely resembles UC and exhibits inflammation that is mostly limited to colonic mucosa [206]. Multiple drug therapies tested on this model have been highly effective. Dexamethasone, a corticosteroid, when administered orally as 3mg/kg dose daily, appeared to inhibit goblet cell loss and leukocyte infiltration in this model [207].Other therapies tested on TCRα$^{-/-}$ mice include immunoglobulin G (with monoclonal auto-antibodies), chitin microparticles administered orally and carbon monoxide. The former can have an impact on B-cell deficient TCRα KO mice [197,208]. Chitin microparticles appear to suppress the production of IL-4 and TNFα and upregulates IFN-γ in mesenteric lymph nodes. The latter has shown to have an anti-inflammatory effect by suppressing IL-1β, IL-4 and TNFα and inducing IL-10 production [209]. Anti-IL-4 antibodies immunotherapy controls Th2-specific cytokine production, thereby suppressing the clinical and histological forms of colitis [197,210].

WASP$^{-/-}$ mice develop spontaneous UC-like colitis at 6 months of age and are used to test the efficacy of WASP-expressing retrovirus [133,197]. Transduction of the virus prior to introducing into WASP-deficient haematopoietic stem cells irradiated mice resulted in the reduction of colitis. The control group of chimeric mice with retrovirus-transduced WASP-deficient hematopoietic cells showed signs of the development of the disease [197,211]. Colitis in *WASP*$^{-/-}$ mice was treated with oral alkaline phosphatase [212,213], neutralisation of Il-4 by weekly anti-IL-4 injections [214] and subsequently a IL-10 immunoglobulin fusion protein completely abrogated colitis in chimeric mice with WASP-deficient immune cells [215].

Mdr1a$^{-/-}$ mice, lacking the multiple drug resistance 1a (Mdr1a) gene, have been useful in testing the efficacy of prophylactic use of broad spectrum antibiotics [197,216]. *Mdr1a*$^{-/-}$ mice, when raised in a germfree environment, are colitis free. The incidence of colitis in specific pathogen free raised *Mdr1a*$^{-/-}$ mice is greatly reduced by the use of broad spectrum antibiotics [216]. Multiple polymorphisms have been identified in the Mdr1a locus in human UC [217].

Targeted therapies were identified to regulate colitis severity in *IL-2*$^{-/-}$ mice. More severe colitis was observed in 2,4,6 trinitrophenol-ovalbumin-immunised *IL-2*$^{-/-}$ mice than in the untreated mice [197]. However, when monoclonal antibodies were administered targeting αEβ7 along with 2,4,6 trinitrophenol-ovalbumin, the result was a reduction of colitis as well as a reduction of $CD4^+$ cells and IFN-γ in the lamina propria [218]. Green tea polyphenol extract in drinking water was used to reduce IFN-γ and TNF-α production, thereby improving the histological features of the spontaneous colitis in *IL-2*$^{-/-}$ [219].

Gαi2$^{-/-}$ mice have been utilised for testing therapeutic agents, resulting in amelioration of colitis. Acellular Bordetella pertussis, when injected into the peritoneal region of Gαi2$^{-/-}$ mice, resulted in upregulation of IL-10 in the gut, subsequently demonstrating a marked reduction in colitis [197,220]. Responsiveness to methyl prednisolone of ex vivo colon cultures of Gαi2$^{-/-}$ mice were alike to those of mice that had been orally treated with the same drug [221]. Ex vivo testing is a promising avenue in validating IBD therapeutic strategies.

The *IL-7 Tg* strain, a model of UC, spontaneously develops acute colitis between 1–3 weeks of age [133,222]. *IL-7* has been identified as a candidate gene correlated with UC and up-regulation of the IL-7 protein expression maintains a long-term effect specifically on CD4+T cells in the mucosa of the colon [223]. This strain has been observed to utilise the IL-7Rα chain in order to orchestrate chronic colitis. This particular chain appears to be expressed only on CD4+ T cells. Blockade of IL-7R efficiently suppresses gut inflammation in adoptive transfer-induced models [224]. *IL-7 Tg* mice are considered suitable for studying T-cell-mediated colitis and are involved in the development of T-cell targeted therapeutics [197].

The TRUC ($Tbet^{-/-}XRag2^{-/-}$) colitis model is useful for investigating mechanisms such as the efficacy of antibiotics, T-regulatory cell infusion and TNF-α neutralisation to ameliorate colitis [225]. TGFβRIIDN displays both spontaneous differentiation of Th cells and multi-organ autoimmune inflammation, particularly IBD [226]. The C3H/HeJBir model develops spontaneous acute colitis, which is usually self-limiting. This model is utilised to assess the particular inhibition of inflammatory mediators and target molecules [227].

Animal models provide the necessary platform for novel treatment strategies [133,197,198]. The choice of relevant mouse models is vital when exploring therapeutic strategies for specific diseases. Current treatments have been associated with immunosuppression to moderate the severity of inflammation and its long-term efficiency is important. Identification of the exact course of events leading to IBD has proved to be challenging as the available colitis models are induced either chemically or immunologically and these modes of induction have revealed contrasting results within

the mouse models. Hence, in the present study a new mouse model, namely Winnie x *Ccr6*$^{-/-}$ mice, was generated and acquired a few interesting and significant findings.

- Genetic ablation of *Ccr6* results in differential expression in Winnie x *Ccr6*$^{-/-}$ colitis.
- *Ccr6* deficiency alters the inflammation pattern in the Winnie x *Ccr6*$^{-/-}$ colon, conferring both exacerbation and amelioration in the colonic segments.

Expression of the genes *Il-1β*, *Il-10*, *Il-23a*, *Il-22*, *Il-6*, *Tgfβ-1*, *Ccr6*, and *Ccl20* were studied in the colonic tissues of Winnie x *Ccr6*$^{-/-}$ mice. Results from the gene expression studies in Winnie x *Ccr6*$^{-/-}$ mice revealed an up-regulation of the gene for pro-inflammatory cytokine IL-1β, in the absence of *Ccr6*, specifically in the proximal colon. Increased *Il-1β* expression in the Winnie x *Ccr6*$^{-/-}$ mouse model is similar to that reported in both UC and CD in humans [145,228]. In the Winnie mice distal colon, *Il-1β* is highly expressed and it was clearly evident from the findings in Winnie x *Ccr6*$^{-/-}$ mice, that *Il-1β* gene expression was highly up-regulated specifically in the proximal colon, in the absence of *Ccr6*. Exacerbation of inflammation in the proximal colon was more pronounced in the Winnie x *Ccr6*$^{-/-}$ phenotype than in the distal colon. Surprisingly, down-regulation of the *Il-1β* gene and reduction in the protein levels in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice reflected the histological outcome, suggesting a preferential production/release in the colonic segments attributed to the absence of *Ccr6*.

With regard to CCR6 ligand expression, we identified an up-regulation of this chemokine ligand, suggesting that the CCL20^{+}Th17 cells are present in the lamina propria of the Winnie x *Ccr6*$^{-/-}$ mice proximal colon in high levels which interestingly revealed opposite patterns to Winnie mice colonic segments. This emphasises that *Ccr*6 is an important

factor in immune regulation, in line with the current literature [91,95]. The expression of *Ccr6* is intrinsically driven by IL-23A and thus an expression of $CCL20^{+}$Th17 cells in the intestinal epithelium and $CCR6^{+}$ Tregs are involved in an intriguing loop of events. Previous studies have established that IL-23 is the key player in a Th17 generation and differentiation and is supposedly linked to *Ccr*6, as it is the surface receptor for Th17 cells and is found in CD [229]. However, in the Winnie x *Ccr6*$^{-/-}$ phenotype, *Il-23a* seemed unaffected by the absence of *Ccr6*. In Winnie x *Ccr6*$^{-/-}$ mice, *Tgfβ1* levels were down-regulated in the proximal and distal colon of Winnie x *Ccr6*$^{-/-}$ mice, and *Il-6* expression was significantly up-regulated, indicating that *Ccr6* is necessary and that it is involved in Th17 recruitment in colitis, concurrent with earlier studies [104].

A significant up-regulation of *Il-22* and *Il-10* expression in the distal colon of Winnie x *Ccr6*$^{-/-}$ mice was identified and reflected the significant increase in IL-10 production in cultured T cells, validating the histological outcome of the phenotype. In the absence of *Ccr6*, the identified amelioration of colitis could possibly be attributed to *Il-10*. Findings from the gene expression, protein levels and clinical experiments all were similar to the histological examination of the distal colon, which mostly remained non-inflamed. The differential expression of Ccr6 was also identified in our immunological findings which clearly showed that there was a down-regulated expression in the $CD4^{+}$ $FOXP3^{+}$ Treg cell population and an up-regulation in the $Ccr6^{+}$ $IL17^{+}$ Th17 population in the draining lymph nodes which implies that in the absence of *Ccr6* the $CD4^{+}$ T cells take up an effector phenotype, given the favourable cytokine milieu, and influences the inflammatory process [52,56,230].

In the absence of *Ccr6* in Winnie x *Ccr6*$^{-/-}$ mice, reduction of $CD4^{+}$ $FOXP3^{+}$ Treg cells indicated that Treg cells choose to facilitate $CD4^{+}$ $IL17^{+}$ T effector cells to accentuate

rather than suppress inflammation in selected sites of the colon, which is attributed to the flexibility in the $CD4^+$T cells. Preferential induction of *Il-10* by lamina propria macrophages and DCs facilitating Th17 cells was reported by previous studies [195] and the same was identified in our findings. *Il-10* was expressed in the distal colon of our model, possibly induced by macrophages and assisting in restricting the inflammation in this part of the colon, suggesting that it could ameliorate if not for *Ccr6*. The decreased number of $CD4^+$ $FOXP3^+$ Treg cells indicated that the $CD4^+$ T cells require *Ccr6* as in its absence the immune cells and innate immunity moves towards augmentation of the disease. The inflammatory variance imparted due to the deficiency of *Ccr6* in the Winnie x *Ccr6*$^{-/-}$ mouse model is remarkable and, to our knowledge, it has not been demonstrated in any other IBD mouse model. The Winnie x *Ccr6*$^{-/-}$ model has, in fact, demonstrated that the expression of $Ccl20^+$ Th17 cells corresponds to the inflammation in being portrayed as independent to *Ccr6*, which needs to be dissected with future experiments in this model and will effectively deliver better understanding with regard to the role of Ccl20 in the absence of *Ccr6*.

The innate immune system plays a very important role in IBD pathogenesis and toll-like receptors (TLRs) play a key role in the mucosal immune system [231]. The regulation of *Ccl20* by the innate immune system is not clearly defined, although the stimulation and release of this ligand into the peripheral blood mononuclear cells from the epithelium have been studied by using TNFα, IL-1α and IL-1β [90,93,232]. Skovdahl et al. [90] demonstrated the up-regulation of TLR3 in IBD, stating that there is an indication that pattern recognition receptors (PRRs) may play a key role in activation of the CCR6-CCL20 axis in IBD. Multi-cytokine mediated inflammation, possibly directed towards a Th17 immune response, was demonstrated in our Winnie x *Ccr6*$^{-/-}$ mice. In the absence of *Ccr6* there exists a differential pattern of expression in the genes involved in the

inflammation process in the Winnie x *Ccr6*$^{-/-}$ phenotype, and further studies are required to explore the biology of the CCR6-CCL20 axis.

The Winnie x *Ccr6*$^{-/-}$ had been generated in order to understand the effects in the absence of the *Ccr6* gene in an existing spontaneous chronic colitis model as Winnie mice display spontaneous distal colitis due to protein misfolding [43]. However, to the contrary, in Winnie x *Ccr6*$^{-/-}$ mice we demonstrated exacerbated proximal colitis. This inflammation pattern was quite intriguing, and the causes of amelioration of distal colitis were unclear. Distribution of inflammation in the colonic segments observed in the Winnie x *Ccr6*$^{-/-}$ mice was a significant finding, and it could possibly be attributed to various events, in the absence of *Ccr6* in a spontaneous colitis model. Differences identified in the immune cell populations of mesenteric lymph nodes in the small intestine and large intestine was reported by Houston et al. [233]. The alterations in the populations with regard to the draining lymph nodes could possibly impact the outcome of the cells analysed from the MLNs.

The obtained results suggest that Winnie x *Ccr6*$^{-/-}$ mice have an increased IL-10 secretion which is thought to be a major contributor to the amelioration of distal colitis. Hence the IL-17/IL-23 pathway in colitis is disrupted and homeostasis is encouraged by the increased production of IL-10 along with the other pro-inflammatory cytokines such as IL-17 and IL-22. Thus homeostasis is conferred in an inflammatory phenotype, resulting in amelioration of distal colitis in Winnie x *Ccr6*$^{-/-}$ mice. The T cell transfer model of colitis, where naïve T cells from *Ccr6*-deficient mice resulted in severe colitis, is the most studied model where it was demonstrated that the IL-10 producing Ccr6^{+} Treg cells, considered novel homing subsets of T cells, were indeed essential in order to confer protection [95].

Development of the *Ccr6*$^{-/-}$ mouse model was vital in understanding the relationship between Treg and Th17 cells, and to identify the immune modulation in the absence of *Ccr6*. Understanding the biological processes involved in the recruitment of T cell subsets and identifying dysregulations in these mouse models is important. *Ccr6*$^{-/-}$ mice have been used to model various autoimmune diseases and immune-mediated diseases [109,234], as they are involved in contrasting roles of both immune regulation and inflammation and, hence, they are used to study IBD pathology [137,140]. *Ccr6*$^{-/-}$ mice exhibited an altered the immune system, displaying a reduction in the Peyer's patches and increased amounts of intraepithelial lymphocytes [49]. When colitis was induced by DSS it resulted in reducing the severity of the pathology and when induced by TNBS it conferred susceptibility to the otherwise resistant strain [87,95]. This evidence alerts us to the fact that in our study the exacerbation of the inflammation indicates that CCR6 is indeed an important factor in the intestinal pathology of colitis in mice.

In a previous study, using the T cell transfer colitis model, the absence of CCR6 resulted in severe colitis and an increased production of IFN-γ producing T cells [95]. Findings from the present study revealed increased IFN-γ producing T cells in the Winnie x *Ccr6*$^{-/-}$ model compared to the *Ccr6*$^{-/-}$ mice, and this suggests that the subsequent inflammation will be severe. However, this study observed regional differentiation and alteration in the inflammation pattern, and hence concludes that Ccr6 is more important to Tregs than Th17cells. Reboldi et al. [235] reported that Ccr6-dependent Th17 cells were recruited into the uninflamed central nervous system (CNS) in the case of induced experimental autoimmune encephalitis (EAE), where Ccl20 was produced in the choroid plexus epithelial cells, which happens to be the site of cerebrospinal fluid production. However, Th17 cells were reportedly the first activated T cell subset which proceeds to initiate a *Ccr6*-independent inflammatory response. This has been challenged by some researchers

who reportedly identified that Th17 cells recruitment was not impacted in *Ccr6*$^{-/-}$ mice CNS, and the disease was thought to be initially delayed, although eventually displayed as an exacerbated clinical EAE [234,236].

Our clinical findings seem to indicate that *Ccr6* does confer exacerbation to the otherwise uninflamed colonic segment. Yamazaki et al. [91] identified that regulation of Ccr6 expression was brought about by TGF-β, along with the transcription factors RORγt and RORα. Th17^{+} Ccr6^{+} Ccl20^{+} cells are induced synergistically by both TGF-β and IL-6 and requiring STAT3, RORγt, RORα and IL-21 in the process [237].

The Th17 subset is considered as a distinct subset of CD4^{+} T cells, producing pro-inflammatory cytokine IL-17 which is associated with the development of inflammation mainly in immune-mediated disorders such as Crohn's disease, RA and psoriasis, along with many others [181,238,239]. Pro-inflammatory cytokines IL-21 and IL-22 are also produced by these CD4^{+} T cells, where IL-21 assists in restoring the balance between Th17 and T reg cells, and IL-22, a unique cytokine belonging to the IL-10 family, is associated with chronic inflammation [240,241]. Differentiation of mouse Th17 cells requires RORC, a transcription factor, in the presence of both IL-6 and TGF-β, further enhanced by TNF and IL-1β [181,242]. Th17 cells can be inhibited by cytokines such as IFN-γ and IL-4 belonging to Th1 and Th2 subsets respectively [243].

It has been widely accepted that TGF-β inhibits most T cell responses and at the same time it is responsible for the induction of FOXP3^{+} Treg cells. The conflicting role of TGF-β was identified and thought to be interesting, as it acts as an inducer of both pro-inflammatory (Th17) and anti-inflammatory (T reg) cells, similar to the intriguing nature of the CCR6-CCL20 [114,117] chemokine axis. The relationship between TGF-β and the T cell subsets needs a better understanding as both Th17 and Treg cells have opposite

roles. Reportedly, in mice, T reg cells act as a basis for Th17 differentiation, while Xu et al. [244] reported that $CD4^+$ $CD25^+$ $FOXP3^+$ T reg cells, in the presence of IL-6, can mould themselves into Th17 and this could be due to the plasticity of $CD4^+$ T cells [52]. It is possible that *Ccr6* modulates this change of T reg cells and enhances the induction of Th17 cells. T reg cells attempt to suppress Th17 cells and seem to be resistant, and this leads us to consider that Tregs could possibly enhance Th17-mediated inflammation [108,181]. Hence, the role of $Ccr6^+$ Treg cells and $Ccr6^+$ Th17 cells in inflammatory diseases need better understanding.

The CCR6-CCL20 axis has been established as being involved in mucosal immunity, although they are reported to be involved in opposing roles. Their involvement in the events of Th17 and T reg cells and B cell differentiation has been extensively researched. The variability in the experimental outcome observed in our study implies that the distal colon needs to be targeted for therapy in order for this to be considered. CCR6 is a potential target for therapy in many autoimmune diseases and the availability of tissue-specific knockout mice is vital. Development of the *Ccl20*$^{-/-}$ mouse model will aid in the understanding of disease pathogenesis. The exact mechanism of CCR6 in IBD remains elusive and the specific role of CCR6-CCL20 in IBD is unclear. Nevertheless, with the aid of research findings from the present study, we conclude that *Ccr6* has the ability to skew the disease progression from inflammation to homeostasis.

6.1 Summary

This study is significant and, to our knowledge, it is the first to examine the role of *Ccr6* in inflammation, associated with a naturally occurring spontaneous colitis in humans. Unlike other contrived models, the Winnie x *Ccr6*$^{-/-}$ model demonstrated proximal colitis due to an SNP simulating a clinical scenario. The present study clearly defines the dichotomy of *Ccr6* in Winnie x *Ccr6*$^{-/-}$ mice colitis. The Winnie x *Ccr6*$^{-/-}$ mice phenotype was explored based on the clinical, histological, molecular and immunological aspects.

Differential patterns of inflammation were observed in regions of the Winnie x *Ccr6*$^{-/-}$ colon. Increased expression of IL-1β indicates an exacerbation of inflammation in the proximal colon to the mid-colon region. The decreased expression of IL-10 suggests amelioration of inflammation in the distal colonic region. Our findings document the clinical and histological changes due to the genetic ablation of *Ccr6* which were supported by molecular and cytokine expressions of both IL-10 and IL-1β. Immunological assessments confirmed the reduction of Ccr6^{+} FOXP3^{+} Treg cells and an increase in the Ccr6^{+} Th17 subpopulation in Winnie x *Ccr6*$^{-/-}$ mice which further suggests that the absence of *Ccr6* mediates a Th17 mucosal response. Distribution of inflammation in the different colonic segments is a key finding in the present study.

6.2 Future directions

There are a few limitations in the present study and hence further studies using larger sample size, isolation of MLNs specific to colonic segments, mucosal enumeration of T regs, along with the other immune cells and evaluation of their functional aspects will be useful in identifying the specific role of *Ccr6* in Winnie x *Ccr6*$^{-/-}$ colitis. In addition,

studies directed towards distribution, localisation of CCL20 and identification of CCR6 as a biomarker can further improve the understanding of the current mouse model, thereby providing vital clues in solving the IBD puzzle. Further research is needed to identify the exact course of events which causes the CCR6-CCL20 chemokine pair to switch between Th17 and Treg cells, specifically in colitis.

From a therapeutic perspective of IBD, the current conventional therapies involve the use of anti-inflammatory drugs such as aminosalicylates, corticosteroids, cyclosporine and biological therapies including monoclonal antibodies. The dendritic cell populations identified in different regions of the draining mesenteric lymph nodes could possibly regulate the outcome of the immune responses and assist in developing tissue-specific therapies. The concept of chemokine-targeted therapy for IBD is now being investigated widely. The CCR6-CCL20 pair is of great interest in autoimmune diseases such as psoriasis, RA and EAE, and is currently being researched as a possible therapeutic target.

In future, immunoregulatory therapy could replace the current immunosuppressive concept, and could potentially open a new myriad of therapeutic target options for IBD. The research findings from our Winnie x *Ccr6*$^{-/-}$ mouse model indicates that precise identification of the *Ccr6* role in colitis will enable a step closer towards a comprehensive understanding of inflammatory bowel disease in humans, which will provide great translational value for future research in the field of IBD.

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APPENDIX

UTAS	University of Tasmania Animal Ethics Committee **ETHICS APPROVAL PERMIT**	University of Tasmania Office of Research Services Ph: 03 62267283 Fax: 03 62267148 animal.ethics@utas.edu.au

To:	Dr Raj Eri
From:	Marilyn Pugsley Executive Officer Animal Ethics
Date:	16 December 2013
Project:	A13691 – Assessment of CCR6/CCl20 as a potential therapeutic target for inflammatory bowel disease
Approved on:	13 December 2013
Approval expires:	13 December 2016
1st Annual Report due:	13 December 2014

Please read this permit carefully as **approval may be withdrawn** for projects that do not comply with the conditions

The Animal Ethics Committee has approved the above project. The approval is subject to the review and approval of an annual report which is due on the approval anniversary. **Please note this date in your diary.**

If the project is to continue past the expiry date, a new initial application will need to be submitted. A project can only be approved for a maximum of 3 years.

As the Responsible Investigator, you MUST ensure that:

(a) all aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

(b) a full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.

(c) you contact the Animal Welfare Officer, Dr Sue Ottomanski (sue.ottomanski@utas.edu.au) to advise her when and where your experiments will be conducted. Sufficient notice needs to be given so that if the AWO wishes to make an inspection, this can be easily arranged.

(d) That all investigators attend Ethics training sessions every three years. Contact the Executive Officer Animal Ethics for the next available session.